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13. ABSTRACT (Maximum 200 Words)

This is a final report of the research carried out from 8/15/1999 to 8/14/2003. Our goal was to study the role of the insulin-like growth factor I receptor (IGF-IR) in breast cancer. IGF-IR is a multifunctional tyrosine kinase activating multiple mitogenic and survival pathways as well as non-mitogenic responses. Current evidence implicates IGF-IR in the development of primary breast tumors. However, the role of IGF-IR in breast cancer metastasis remains unknown. During the course of this work, we studied IGF-IR-dependent phenotype in ER-positive (less metastatic) and ER-negative (more metastatic) breast cancer cells. We focused on the involvement of IGF-IR in cell migration and adhesion, the processes that regulate the development of metastasis.

We found that in ER-positive breast cancer cells, IGF-IR not only promotes cell growth and survival but also upregulates cell-cell adhesion by strengthening E-cadherin-dependent cell junctions. IGF-IR-increased cell-cell adhesion improved cell survival, decreased the sensitivity of cells to the antiestrogen ICI 182,780 and reduced invasiveness in organ culture. On the other hand, in ER- and E-cadherin-negative breast cancer cells, IGF-IR was not able to promote cell growth, survival, and cell-cell adhesion, but was acting as a chemoattractant stimulating cell motility. The molecular mechanisms of IGF-IR-regulated growth, survival, adhesion, and migration, and the differences in IGF-IR action in ER-positive and ER-negative breast cancer cells have been detailed by us in several peer-reviewed publications (3 original papers and 3 reviews).

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Appendix: Manuscripts:

Surmacz, E. Growth factor receptors as therapeutic targets: Strategies to inhibit the insulin-like growth factor I receptor. Oncogene, in press, 2003.

Morelli, C., Garofalo, C., Bartucci, M., Surmacz, E. Estrogen receptor-alpha regulates the degradation of the insulin receptor substrates 1 and 2 in breast cancer cells. Oncogene, 22: 4007-4016, 2003.

Mauro, L., Salerno, M., Morelli, C., Boterberg, T., Bracke, M., Surmacz, E. Role of the IGF-I receptor in the regulation of cell-cell adhesion. Implications in cancer development and progression. J.Cell. Physiol. 194: 108-116, 2003.

Mauro, L., Bartucci, M., Morelli, C., Ando', S., Surmacz, E. IGF-I receptor-induced cell-cell adhesion of MCF-7 breast cancer cells requires the expression of junction protein ZO-1. J. Biol. Chem. 276: 39892-39897, 2001.

Bartucci, M., Morelli, C., Mauro, L., Ando', S., Surmacz, E. Differential insulin-like growth factor I receptor signaling and function in estrogen receptor (ER)-positive MCF-7 and ER-negative MDA-MB-231 breast cancer cells. Cancer Res. 61: 6747-6754, 2001.

Surmacz, E. Function of the IGF-IR in breast cancer. J. Mammary Gland Biol. Neopl., 5: 95-105, 2000.

INTRODUCTION

The insulin-like growth factor I (IGF-I) receptor (IGF-IR) is a ubiquitous multifunctional tyrosine kinase. IGF-IR regulates normal breast development; however, hyperactivation of the same receptor has been implicated in breast cancer (1). Laboratory studies demonstrated that IGF-IR stimulates the growth and survival in several breast cancer cell lines and counteracts the effects of various anti-tumor treatments (1). The analysis of clinical specimens suggested that overexpression of either IGF-IR or its major signaling substrate IRS-1 in estrogen receptor alpha (ER)-positive breast tumors has been linked with cancer recurrence at the primary site (1). Furthermore, high circulating levels of IGF-I (ligand of IGF-IR) have been associated with increased breast cancer risk in premenopausal women (1).

Although current evidence suggests that abnormal activation of IGF-IR may contribute to the autonomous growth and increased survival of primary ER-positive breast tumors, the function of this receptor in breast cancer metastasis is not clear. For instance, some small clinical studies demonstrated a correlation between IGF-IR expression in node-positive tumors and worse prognosis. Other data linked IGF-IR expression with better clinical outcome, as IGF-IR was predominantly expressed in a subset of breast tumors with good prognostic characteristics (1). In the experimental setting, anti-IGF-IR strategies were successfully applied to inhibit the growth and spread of human breast cancer xenografts, which implicated IGF-IR in metastasis (1). Thus, understanding whether hyperactivation of IGF-IR could be a factor promoting breast cancer metastasis is of great importance.

Our previous data demonstrated that overexpressed IGF-IRs not only promote breast cancer cells growth, but also activate aggregation and prolong survival of detached cells (2). This process was blocked with a specific antibody against E-cadherin (E-cad)—a major cell-cell adhesion protein expressed by epithelial cells, suggesting that the E-cad complex mediates IGF-IR effects (2, 3). The molecular mechanism of IGF-I-induced E-cad-dependent adhesion and the significance in the process of metastasis have been unknown. Consequently, our goal was to study the effects of IGF-I on the expression of E-cad and E-cad-associated proteins and to determine the interactions of IGF-IR with these adhesion proteins. Moreover, we set out to evaluate the importance of the IGF-IR/E-cad interactions in two processes involved in breast cancer progression: antiestrogen resistance, migration, and invasion. In addition, because during breast cancer progression both ER and E-cad are often lost or downregulated, we probed how IGF-IR signaling and function can be affected by the lack of interactions with the ER and E-cad systems.

TECHNICAL REPORT

This is a final report of research carried out in Years 1-4.

Project I: IGF-IR interactions with the E-cad complex.
The results summarized below are detailed in Mauro et al. 2001, 2003 (Ref. 3 and 4), reprints provided in Appendix

The original application was based on our preliminary observations that ER-positive MCF-7 breast cancer cells overexpressing IGF-IR exhibit increased aggregation in 3-dimensional (3-D) culture (2). Our results implicated that IGF-IR-induced adhesion is mediated through the

E-cad adhesion complex (2). Subsequent studies supported by this award demonstrated that the levels of E-cad and its associated proteins (alpha-, beta-, and gamma-catenin) were not affected by IGF-IR overexpression (4). However, the levels of ZO-1, a scaffolding protein linking E-cad through alpha-catenin to the actin cytoskeleton, were significantly increased in cells overexpressing IGF-IR (MCF-7/IGF-IR) (4). Additional experiments demonstrated that ZO-1 mRNA was overexpressed in MCF-7/IGF-IR cells relative to MCF-7 cells (4). Further analysis of interactions of IGF-IR with adhesion proteins revealed that IGF-IR co-precipitates and co-localizes with E-cad and ZO-1 (3, 4). IGF-IR and ZO-1 also associated with alpha-catenin. The amounts of IGF-IR/ZO-1/alpha-catenin complexes were greater in MCF-7/IGF-IR then in MCF-7 cells (3). Cumulatively, these results suggested that IGF-IR overexpression or activation upregulates ZO-1, which in turn leads to the strengthening of E-cad adhesion (4).

To confirm the observation that ZO-1 expression is necessary for cell-cell adhesion induced by the IGF-IR, we employed anti-ZO-1 antisense strategy. We developed anti-ZO-1 antisense RNA construct and subsequently we created, by stable transfection, MCF-7/IGF-IR/anti-ZO-1 cell lines. In these clones, the levels of ZO-1 were downregulated, whereas the expressions of E-cad and IGF-IR were unchanged. The lower levels of ZO-1 in MCF-7/IGF-IR/anti-ZO-1 cells coincided with reduced cell-cell adhesion (4).

After we elucidated the mechanism of IGF-IR-induced cell-cell adhesion, we studied biological consequences of this phenomenon. We focused on two processes important in breast cancer progression: antiestrogen resistance and invasion. In the studies of antiestrogen resistance, we employed MCF-7 cells and MCF-7 cells overexpressing the IGF-IR (MCF-7/IGF-IR cells). The cells were grown in three-dimensional (3-D) culture. Under these conditions, MCF-7 cells form small (~50 um in diameter) spheroids, while MCF-7/IGF-IR cells aggregate into large (~300 um) spheroids. Both MCF-7 and MCF-7/IGF-IR spheroids are viable for several days, and MCF-7/IGF-IR spheroids may even increase in size and cell number during culture (2). Characteristically, the IGF-IR is constitutively activated in MCF-7/IGF-IR spheroid cultures (4).

ICI 182,780, a pure antiestrogen, reduced the survival of MCF-7 spheroids by ~50%, but had no effect on MCF-7/IGF-IR spheroids. However, when MCF-7/IGF-IR aggregates were disrupted with EGTA or anti-E-cad antibody, ICI 182,780 was able to reduce the number of cell (3). This suggested that IGF-IR-dependent cell-cell adhesion may represent a mechanism of enhanced resistance to antiestrogen treatment. Our preliminary results indicated that despite higher tyrosine phosphorylation of IGF-IR in MCF-7/IGF-IR spheroids, in both cell models, the Akt survival pathway was activated to a similar extent under basal conditions and was comparably inhibited by ICI 182,780 treatment (not shown).

Next, we probed the effect of IGF-IR-induced cell-cell adhesion on the invasive potential of breast cancer cells. We used MCF-7/6 cells that are characterized by a moderate invasive potential. The cells contain IGF-IR and express several elements of the E-cad complex (3, 5, 6). It has previously been demonstrated that IGF-I treatment decreased invasiveness of these cells through an E-cad-dependent mechanism (5, 6). Using precultured chick heart fragments (PHF) we investigated whether overexpression of IGF-IR in MCF-7/6 cells modifies invasiveness. Two MCF-7/6/IGF-IR clones A6 and A9, obtained by stable transfection of IGF-IR cDNA, were studied. The cells were first aggregated and then cultured in contact with PHF for several days. The degree of breast cancer cell invasion into PHF was evaluated by histology. The overexpression of IGF-IR significantly decreased invasiveness of MCF-7/6 cells

bringing it to the levels comparable to that seen in MCF-7/AZ cells (non-invasive control, similar to MCF-7 cells) (3).

Our observations on anti-invasive role of IGF-IR in MCF-7 cells were confirmed by another group using MCF-7 cells with reduced levels of IGF-IR. Specifically, decreasing the levels of IGF-IR resulted in reduced cell-cell adhesion and enhanced migration and invasion (7).

Cumulatively, our data suggest that in ER-positive, E-cad-positive breast cancer cells, activation of IGF-IR promotes cell adhesion, which enhances cell survival and resistance to antiestrogen treatment, but reduces cell invasion. This concurs with the results obtained with tumor specimens where IGF-IR was found be co-expressed with ER and other good prognostic markers (1).

Project II: Regulation of cell-cell adhesion by E2/IGF-I cross-talk

E2 treatment improves IGF-IR-induced cell-cell adhesion.

Because the expression of E-cad correlates with ER-positive status, we assessed the effects of E2 on cell-cell adhesion. The treatment with estradiol (E2) effectively stimulated cell aggregation in 3-D culture and potentiated IGF-I effects, as measured by the number of large spheroids [Tab.1]. The effect of E2 was blocked with the antiestrogen 182,780 [Tab. 1] and required E-cad, as it was blocked with anti-E-cad Abs (data not shown), but it was not related to increased expression of IGF-I or ZO-1 [Fig. 1]. The latter indicated that the mechanism of the stimulation of cell-cell adhesion by E2 is different that that observed with IGF-I. Currently, the studies are ongoing in our laboratory to determine the regulation of the proteins within the E-cad complex by E2, and to define the molecular pathways of E2- and IGF-I-dependent cell-cell adhesion.

Cells/Treatments	Spheroids		
	25 < 50 μm	50 < 100 μm	> 100 μm
MCF-7	11.0±2.2	89±9.0	0±0.0
MCF-7 + IGF-I	0.0±0.0	29±3.9	64±12.1
MCF-7 + E2	5.9±3.5	40.7±2.1	45±12.9
MCF-7 + IGF-I + E2	0.0±0.0	10.0±6.5	109.9±0.0
MCF-7 + IGF-I + ICI 182,780	8.0±2.0	80.5±5.0	0.0±0.0

Tab. 1. Effects of E2 on cell aggregation in E-cad-positive breast cancer cells. The cells were grown to 70-80% confluence, trypsinized, and plated as single cell suspension in 2%-agar-coated plates at the concentration $2x10^6$ cells per 100 mm culture dish. The cells were either left untreated in SFM or were treated with 50 ng/ml IGF-I (IGF-I), 10 nM E2 (E2), 50 ng/nl IGF-I and 10 nM E2 (IGF-I + E2). ICI 182,780 was used at 100 uM. To generate 3-D spheroids, the plates were rotated for 4 h at 37°C. The spheroids started to assemble at ~1 h after plating and were completely organized after 3-4 h of culture in suspension. The 3-D cultures were observed using a phase contrast microscope (Nikon or Olympus). The extent of aggregation was scored at 1 day of treatment by measuring the spheroids with an ocular micrometer. For each cell type, the spheroids of 25≤50, 50≤100, and >100 μm (in the smallest cross-section) were counted in 10 different fields under 10x magnification.

Fig. 1. Effects of E2 on ZO-1 and IGF-IR expression in MCF-7 cells in 3-D culture. The expression of ZO-1 and IGF-IR was studied by Western blotting in 50 ug of protein lysates obtained from cells cultured as 3-D spheroids in SFM, SFM plus 10 nM E2, SFM plus 50 ng/ml IGF-I, or SFM plus 10 nM E2 and 50 ng/ml IGF-I for 24 h. Anti-IGF-IR mAb (Calbiochem) and anti-ZO-1 mAb (Zymed) were used as recommended by the manufacturer.

Project III: Function of IGF-IR in ER-negative, E-cad-negative breast cancer cells.
The results summarized below are detailed in Bartucci et al. 2001 (Ref. 8), reprint provided in Appendix

The function of IGF-IR has also been addressed in E-cad-negative, ER-negative breast cancer cells. ER-negative breast cancer cells often express low levels of the IGF-IR and fail to respond to IGF-I with mitogenesis (1). On the other hand, anti-IGF-IR strategies effectively reduced metastatic potential of different ER-negative cell lines, suggesting a role of this receptor in late stages of the disease (1).

We examined IGF-IR signaling and function in ER-negative MDA-MB-231 breast cancer cells and their IGF-IR-overexpressing derivatives (8). We demonstrated that IGF-I acts as a chemoattractant for these cells. The extent of IGF-I-induced migration reflected IGF-IR levels and required the activation of PI-3K and p38 kinases. In contrast with the positive effects on cell migration, IGF-I was unable to stimulate growth or improve survival in MDA-MB-231 cells, while it induced mitogenic and anti-apoptotic effects in control MCF-7 cells. Overexpression of IGF-IR also did not promote cell-cell adhesion in ER- and E-cad-negative MDA-MB-231 cells (4). The impaired IGF-I growth response of ER-negative cells was not caused by the low IGF-IR expression, defective IGF-IR tyrosine phosphorylation, or improper tyrosine phosphorylation of IRS-1, but mapped downstream of IRS-1 (8). Detailed analysis and time course experiments indicated that the defect is caused by the inability of cells to sustain the activation of PI-3K/Akt pathway. Our results suggested that IGF-IR growth/survival-related functions may depend on ER expression, but non-mitogenic activities such as migration can be initiates regardless of ER status.

Key Research Accomplishments:

- Demonstrated differential function and signaling of IGF-IR in ER-positive and ER-negative breast cancer cells.
- Demonstrated that strong cell-cell adhesion induced by the activated IGF-IR in ER-positive, E-cad-positive breast cancer cells reduced sensitivity of cells to antiestrogens and inhibited cell invasion in organ culture.
- Demonstrated that in ER-negative, E-cad-negative breast cancer cells, IGF-IR is not mitogenic but retains the ability to stimulate migration.
- Provided evidence that IGF-IR can become a pharmaceutical target in breast cancer, especially in ER-positive tumors.

Reportable Outcomes:

1. Manuscripts, abstracts and scientific presentations:

Manuscripts:

Mauro, L., Surmacz, E. IGF-I receptor, cell-cell adhesion, tumor development and progression.Histochem. J., submitted, 2003.

Surmacz, E. Growth factor receptors as therapeutic targets: Strategies to inhibit the insulin-like growth factor I receptor. Oncogene, in press, 2003.

Morelli, C., Garofalo, C., Bartucci, M., Surmacz, E. Estrogen receptor-alpha regulates the degradation of the insulin receptor substrates 1 and 2 in breast cancer cells. Oncogene, 22: 4007-4016, 2003.

Mauro, L., Salerno, M., Morelli, C., Boterberg, T., Bracke, M., Surmacz, E. Role of the IGF-I receptor in the regulation of cell-cell adhesion. Implications in cancer development and progression. J.Cell. Physiol. 194: 108-116, 2003.

Mauro, L., Bartucci, M., Morelli, C., Ando', S., Surmacz, E. IGF-I receptor-induced cell-cell adhesion of MCF-7 breast cancer cells requires the expression of junction protein ZO-1. J. Biol. Chem. 276: 39892-39897, 2001.

Bartucci, M., Morelli, C., Mauro, L., Ando', S., Surmacz, E. Differential insulin-like growth factor I receptor signaling and function in estrogen receptor (ER)-positive MCF-7 and ER-negative MDA-MB-231 breast cancer cells. Cancer Res. 61: 6747-6754, 2001.

Surmacz, E. Function of the IGF-IR in breast cancer. J. Mammary Gland Biol. Neopl., 5: 95-105, 2000.

Abstracts (Presentations and Invited Talks):

Surmacz, E., Morelli, C., Sisci, D., del Rincon, S., Garofalo, C. Estrogen stimulates nuclear translocation of IRS-1 and IRS-1 modulates estrogen receptor-dependent transcription in breast cancer cells. IV International Symposium on Hormonal Carcinogenesis, Valencia, Spain, July 22-25, 2003.

Surmacz, E., Morelli, C., Sisci, D., del Rincon, S., Garofalo, C. Estrogen stimulates nuclear translocation of IRS-1 and IRS-1 modulates estrogen receptor-dependent transcription in breast cancer cells. The Endocrine Meeting, Philadelphia, Pa, July 18-22, 2003 (speaker).

Mauro, L., Morelli, C., Bartucci, M., Ando, S., Surmacz, E. The mechanism of IGF-I receptor-induced cell-cell adhesion of MCF-7 breast cancer cells. Era of Hope DOD Breast Cancer Program Meeting, Orlando, FL, September 25-28, 2002

Surmacz, E. New aspects of IGF-I receptor signaling in ER-positive and ER-negative breast cancer cells. AstraZeneca, Macclesfield, U.K., August 8, 2002 (invited speaker).

Morelli, C., Bartucci, M., Garofalo, C., Surmacz, E. Role of estrogen receptor-alpha in the stability of insulin receptor substrates 1 and 2. Endocrinology Meeting, San Francisco, CA June 22-26, 2002.

Surmacz, E. IGF-I signaling in ER-positive and ER-negative breast cancer cells. Temple University, Center for Neurovirology and Tumor Biology, Philadelphia, PA, April 11, 2002 (invited speaker).

Surmacz, E. Is IGF-IR involved in breast cancer? Oncology Group Meeting. Thomas Jefferson University, Philadelphia, Pa, June 28, 2001 (invited speaker).

Salerno, M., Mauro, L., Panno, M. L., Belizzi, D., Sisci, D., Miglietta, A., Surmacz, E., Ando', S. Estradiol enhance IRS-1 gene expression and amplifies insulin signaling in ER-positive breast cancer cells. The Endocrine Society 83rd Annual Meeting. Denver, Co, June 20-23, 2001.

Surmacz, E. Differential IGF-I signaling in ER-positive and ER-negative breast cancer cells. Department of Radiology. University of Pennsylvania, Philadelphia, PA, May 16, 2001 (invited speaker).

Surmacz, E. IGF signaling in ER-negative breast cancer cells. Postgraduate School in Molecular Pathology. University of Calabria, Italy, May 11, 2001 (invited speaker).

Surmacz, E. IGF-I receptor/estrogen receptor cross-talk in breast cancer. Postgraduate School in Molecular Pathology. University of Calabria, Italy, May 10, 2001 (invited speaker).

Surmacz, E. Differential IGF-I receptor signaling in ER-positive and ER-negative breast cancer cells. Kimmel cancer Center, Staff Seminar Series, Philadelphia, PA, April 9, 2001 (speaker).

Surmacz, E. Differential IGF-I receptor signaling in ER-positive and ER-negative breast cancer cells. Temple University, Center for Neurovirology and Tumor Biology, Philadelphia, PA, April 5, 2001 (invited speaker).

Surmacz, E. Role of the E-cadherin complex in breast cancer cell adhesion. Philadelphia Cadherin Club Meeting. Lankenau Cancer Research Center, Wynnewood, PA, March 1, 2001 (invited speaker).

Surmacz, E. Differential IGF-IR function in ER-positive and ER-negative breast cancer cells. AstraZeneca, Macclesfield, U.K., September 18, 2000 (invited speaker)

Surmacz, E. Evolution of IGF-IR signaling during breast cancer progression. "IGFs and Cancer" International Symposium, Halle, Germany, September 14-17, 2000 (invited speaker).

Morelli, C., Bartucci, M., Mauro, L., Ando' S., Surmacz, E. Insulin-like growth factor I receptor (IGF-IR) signaling in metastatic breast cancer cells. The Endocrine Society Annual Meeting, Toronto, Canada, June 21-24, 2000.

Surmacz, E. Insulin-like growth factor I signaling is regulated by estrogen receptor in breast cancer cells. Era of Hope, Department of Defense Breast Cancer Research Program Meeting, Atlanta, June 8-11, 2000.

Bartucci, M., Mauro, L., Salerno, M., Morelli, C., Ando', Surmacz, E. Function of the insulin-like growth factor I receptor in metastatic breast cancer cells. Era of Hope, Department of Defense Breast Cancer Research Program Meeting, Atlanta, June 8-11, 2000.

Surmacz, E. Is IGF-IR involved in breast cancer? Lankenau Cancer Research Center, Wynnewood, PA, February 3, 2000 (invited speaker).

Surmacz, E. Insulin treatment and breast cancer. Novo Nordisk Pharmaceuticals. Princeton, NJ., January 25, 2000 (invited speaker).

Surmacz, E. IGF-IR and breast cancer. Department of Cellular Biology, University of Calabria, Cosenza, Italy, December 9, 1999 (invited speaker)

Bartucci, M., Mauro, L., Salerno, M., Morelli, C., Ando', S., Surmacz, E. Function of the insulin-like growth factor I receptor in metastatic breast cancer cells. 22nd Annual Breast Cancer Symposium. San Antonio, TX, December 8-11, 1999

Surmacz, E., Mauro, L., Ando', S. New insights into IGF-I-dependent regulation of cell-cell adhesion in breast cancer cells. 5th International Symposium on IGFs. Brighton, U.K., October 31-November 4, 1999 (speaker)

Surmacz, E. IGF-IR signaling in breast cancer. University of Gent, Gent, Belgium, September 29, 1999 (invited speaker)

2. Patents and licenses: None

3. Degrees: N/A

4. Development of biologic reagents: None

5. Databases: None

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2004-2009	NIH PO-1 "Cell Cycle Progression" Project 3 "New Aspects of IGF-I/ER Crosstalk in Breast Cancer" $122,050/yr, Project 3 Leader (PI A. Giordano). Pending
2004-2009	NIH RO-1 "New interactions in ER/IGF-I crosstalk" $200,000/yr, PI. Pending

7. Employment applied for: None

Conclusions

IGF-IR hyperactivation can improve cell survival and induce drug resistance in ER-positive breast cancer cells. In part, this effect of IGF-IR is mediated through increased cell-cell adhesion. Thus, IGF-IR could become a viable pharmaceutical target in ER-positive, E-cad-positive breast tumors.

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Function of the IGF-I Receptor in Breast Cancer

Eva Surmacz[1,2]

The insulin-like growth factor-I receptor (IGF-IR)[3] is a transmembrane tyrosine kinase regulating various biological processes such as proliferation, survival, transformation, differentiation, cell-cell and cell-substrate interactions. Different signaling pathways may underlie these pleiotropic effects. The specific pathways engaged depend on the number of activated IGF-IRs, availability of intracellular signal transducers, the action of negative regulators, and is influenced by extracellular modulators. Experimental and clinical data implicate the IGF-IR in breast cancer etiology. There is strong evidence linking hyperactivation of the IGF-IR with the early stages of breast cancer. In primary breast tumors, the IGF-IR is overexpressed and hyperphosphorylated, which correlates with radio-resistance and tumor recurrence. *In vitro,* the IGF-IR is often required for mitogenesis and transformation, and its overexpression or activation counteract effects of various pro-apoptotic treatments. In hormone-responsive breast cancer cells, IGF-IR function is strongly linked with estrogen receptor (ER) action. The IGF-IR and the ER are co-expressed in breast tumors. Moreover, estrogens stimulate the expression of the IGF-IR and its major signaling substrate IRS-1, while antiestrogens downregulate IGF-IR signaling, mainly by decreasing IRS-1 expression and function. On the other hand, overexpression of IRS-1 promotes estrogen-independence for growth and transformation. In ER-negative breast cancer cells, usually displaying a more aggressive phenotype, the levels of the IGF-IR and IRS-1 are often low and IGF is not mitogenic, yet the IGF-IR is still required for metastatic spread. Consequently, IGF-IR function in the late stages of breast cancer remains one of the most important questions to be addressed before rational anti-IGF-IR therapies are developed.

KEY WORDS: Breast cancer; insulin-like growth factor I receptor; IRS-1; estrogen-independence; antiestrogen; metastasis.

INTRODUCTION

The insulin-like growth factors I and II (IGFs) act as endocrine, paracrine or autocrine regulators of various biological processes in normal and neoplastic cells. The actions of IGF-I in the adult are mediated primarily by the type I insulin-like growth factor receptor (IGF-IR), while IGF-II stimulates both the IGF-IR and the insulin receptor (IR) (1,2). It has been well established that in many cell types, activation of the IGF-IR is essential for cell survival, transformation, and hormone-independence—the processes that pro-

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[3] Abbreviations: three-dimensional (3-D); amino acid; (aa); disease-free survival (DFS); E-cadherin (E-cad); 17-beta estradiol (E2); extracellular matrix (ECM); epidermal growth factor receptor (EGFR); estrogen receptor (ER); histidine (His); insulin-like growth factor (IGF); IGF-I receptor (IGF-IR); insulin receptor (IR); insulin receptor substrate (IRS); lysine (Lys); overall survival (OS); phosphatidylinositol-3 kinase (PI-3K); progesterone receptor (PgR); protein kinase C (PKC); phosphotyrosine binding domain (PTB); MCF-7 cells expressing anti-IRS-1 and anti-SHC RNA, respectively (MCF-7/anti-IRS-1 and MCF-7/anti-SHC); MCF-7 cells overexpressing the IGF-IR, IRS-1, and SHC, respectively (MCF-7/IGF-IR, MCF-7/IRS-1, and MCF-7/SHC); serine (Ser); src-homology 2 domain (SH2); src/collagen homology proteins (SHC); Tamoxifen (Tam).

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mote tumorigenesis (3–6). During the past several years, the impact of the IGF-IR on breast cancer development and progression has also been recognized, providing a new direction for the design of anti-growth factor compounds for breast cancer therapy.

IGF-IR EXPRESSION AND STRUCTURE

Almost all cell types, except hepatocytes and T-lymphocytes, express the IGF-IR (1,3). The IGF-IR is encoded by a 100 kb gene containing 21 exons located on the distal arm of chromosome 15 (1,7). The IGF-IR promoter region is GC-rich, and similar to other housekeeping genes lacks TATA or CCAAT boxes, but contains several sites for binding transcriptional factors such as SP-1, E2F, and early growth response (EGR) proteins (5,8,9). The expression of the IGF-IR is regulated by different physiologic stimuli and may be altered in certain pathologies (e.g., diabetes, cancer). For instance, IGF-IR mRNA is enhanced by growth hormone, follicle stimulating hormone, luteinizing hormone, thyroid hormones, glucocorticoids, and estrogens (9). Moreover, different mitogens (e.g., platelet-derived growth factor, fibroblast growth factor) or oncoproteins (e.g., c-myb, hepatitis B Hbx) can induce IGF-IR transcription. Conversely, IGF-IR expression is downregulated by high concentrations of IGF-II, interferon, antiestrogens, and tumor supressors (e.g., Wilms' tumor or p53 proteins) (4,8,9).

The major IGF-IR 11 kb transcript is translated into a single 1,367 amino acid (aa) (180 kDa) precursor protein, which is then cleaved to form 135 kDa alpha and 90 kDa beta subunits. A mature IGF-IR is a heterotetramer composed of two alpha and two beta subunits linked by disulfide bonds (Fig. 1). The extracellular alpha subunits are responsible for ligand binding. IGF-IR beta subunits, which contain short transmembrane and large intracellular segments, transmit ligand-induced signal (1,7,9). Within the beta subunit, three major domains have been recognized: a tyrosine kinase domain, a juxtamembrane part, and the C-terminus, each containing residues essential for different IGF-IR functions (Fig. 1). Specifically, in the kinase domain, the ATP binding site containing lysine (Lys) 1003 as well as the tyrosine (Tyr) cluster (Tyr 1131, 1135, 1136) are critical for the catalytic activity of the receptor (5,9,10). In the juxtamembrane domain, Tyr 950 flanked by the NPEY motif is required for recruiting major signaling substrates such as insulin receptor substrates (IRS) 1-4 and src/collagen-homology (SHC) proteins (5,6,9,10). The C-terminus contains several residues essential for IGF-I signaling, including Tyr 1250, Tyr 1251, a stretch of serines (Ser) 1280-1283, histidine (His) 1293, Lys 1294, and Tyr 1316. In particular, the region between residues 1229 and 1245 has been found necessary for the association of an adapter GRB10, Tyr 1251 is required for binding a putative substrate p28, Ser 1280-1283 are necessary to sequester an adapter 14-3-3 epsilon, and Tyr 1316 is capable of recruiting either p85 subunit of phosphatidyl inositol-3 kinase (PI-3K) or SHPTP2 phosphatase (5,9,10, Baserga *et al.*, unpublished data). According to recent evidence, Tyr 1251 also appears to be indirectly involved in binding of SHC to the IGF-IR (11).

The IGF-IR shares significant structural homology with the IR. The kinase domains of these receptors are 80–90% identical. Also, Tyr 950 of the IGF-IR has its equivalent, Tyr 960, in the juxtamembrane domain of the IR (7). Importantly, the C-terminal regions of the receptors are quite different, sharing only approximately 40% homology. The equivalents of Tyr 1250 and 1251, Ser 1280–1283, and aa 1293–1301 are not present in the IR. Consequently, it is believed that the differences between biological responses of the IGF-IR and IR are associated with the induction of specialized signaling pathways arising from the C-terminus (1,7,10).

IGF-IR SIGNALING

Upon ligand binding, IGF-IRs cluster and tyrosine kinase is activated leading to autophosphorylation and transphosphorylation of beta subunits (1). Phosphorylation of specific Tyr and Ser residues creates binding sites for IGF-IR signaling substrates. The best known substrates are docking proteins IRS-1 and SHC. Both bind Tyr 950 through their phosphotyrosine binding (PTB) domain (4,9).

IRS-1 is a remarkable effector of the IGF-IR, capable of amplification and diversification of the signal because it can recruit various signaling molecules and induce numerous cellular responses. IRS-1 contains about 20 tyrosine phosphorylation sites which can directly bind signaling molecules equipped with phosphotyrosine binding domains, such as src-homology 2 (SH2) domains. For instance, there are nine YMXM motifs in IRS-1 that can attract the p85 subunit of PI-3K via SH2-type interactions and other domains recruiting SH2-containing adapters GRB2, Nck, and Crk, SHP2 phosphotyrosine phosphatase, and Fyn

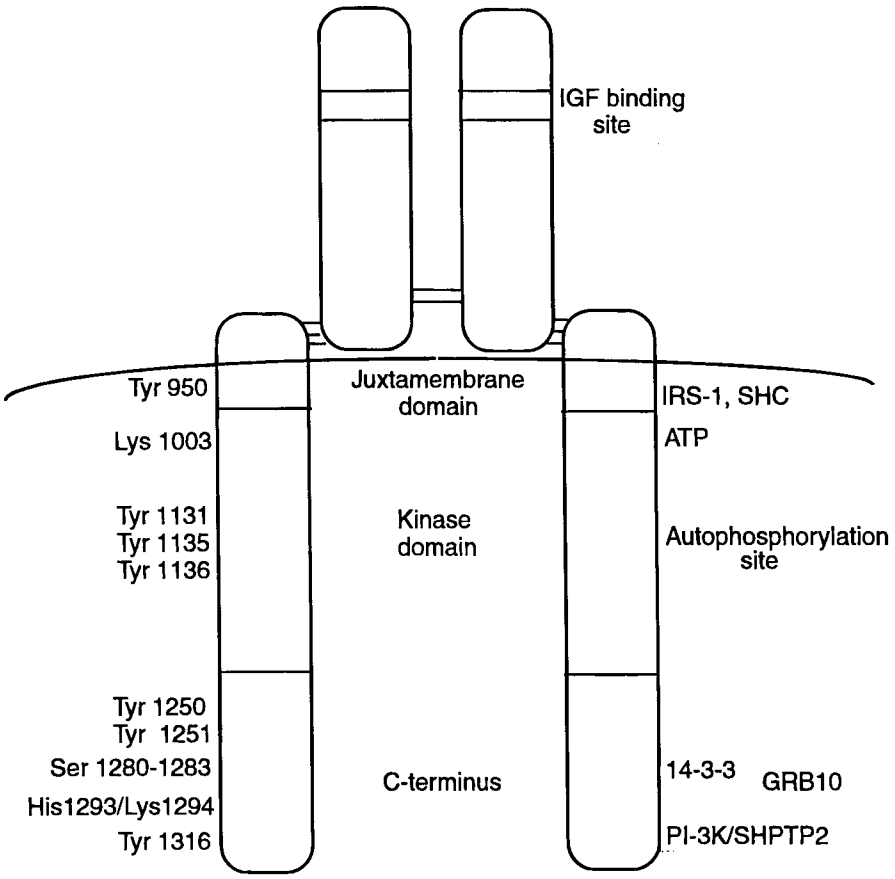

Fig. 1. Structure of the IGF-IR. The key residues involved in IGF-IR signaling are indicated on the left; the signaling elements binding to these regions of the IGF-IR are listed on the right.

kinase. Other partners of IRS-1, such as integrin $\alpha v\beta 3$ or the adapter 14-3-3, associate with the substrate through unknown mechanisms (12).

The major pathway induced by tyrosine phosphorylation of IRS-1 involves PI-3K, whose downstream effectors are Ser/threonine kinases Akt, $p70^{S6}$, and some isoforms of protein kinase C (PKC) (13). PI-3K is involved in the regulation of mitogenesis, metabolism, and actin cytoskeleton rearrangements, and the PI-3K/Akt pathway has recently been recognized as one of the most important signals ensuring cell survival. One of the cellular targets of Akt is a pro-apoptotic protein BAD, which induces cell death when bound to anti-apoptotic proteins Bcl2 or Bcl_{XL}. The phosphorylation of BAD by Akt facilitates its sequestration by 14-3-3 adapters and prevents apoptosis. Another effector of Akt is $p70^{S6}$ kinase which activates expression of cyclin D1 initiating cell cycle progression (13,14).

Association between IRS-1 and the GRB2/SOS complex leads to the stimulation of the classic Ras/MAP cascade of kinases, the pathway that is implicated in a broad array of biological responses, including cell growth and differentiation (12,15). The Ras/MAP pathway can also be induced through Nck or Crk adapters binding to IRS-1 or by GRB2 binding to IGF-IR-associated SHC proteins (6,12,15).

Three additional IRS proteins (IRS-2, -3, and -4) exhibiting different degrees of structural homology to IRS-1 have been cloned. The activities of IRS-1 and IRS-2 appear to partially overlap; for instance, in IRS-1-knock-out mice, IRS-2 substituted for IRS-1 function in stimulating PI-3K and activating glucose metabolism. However, in IRS-1-deficient fibroblasts, only IRS-1, but not IRS-2, reconstituted cell cycle progression (12,13). The functions of IRS-3 and -4 are not well understood. Similarly, the pathways initiated by

the binding of GRB10 or 14-3-3 to the IGF-IR are still obscure.

IGF-IR SIGNAL SPECIFICITY

Because the IGF-IR may regulate many, often contradictory (e.g., growth vs. differentiation), processes, it is of great importance to understand how signal specificity is achieved. Here, I will focus on IGF-IR-dependent survival, mitogenesis, and anchorage-independent growth, the most studied IGF effects. The current view is that the IGF-I response is dictated by the engagement of different sets of intracellular pathways. Which pathways are stimulated depends on (i) the number of activated receptors on the cell surface; (ii) the availability of signaling substrates and receptor-substrate binding sites; (iii) the abundance and activity of negative regulators such as phosphatases; and (iv) extracellular context, e.g., ligand availability or extracellular matrix (ECM) components and their interaction with cells.

The first point is best illustrated by the work of Rubini *et al.* (16) and Reiss *et al.* (17), who analyzed the relationship between the number of IGF-IRs expressed on the cell and IGF-I-induced biological response. While activation of $2–10 \times 10^3$ receptors stimulates tyrosine phosphorylation of IRS-1 as well as activation of an early response gene *c-myc*, it is not sufficient for SHC phosphorylation or the entry of cells into the cell cycle. With 1.5×10^4 receptors, the cells progress through the S phase, but they are not able to complete cell division, and their survival ability under anchorage-independence is minimal. The increase of receptor expression to $2.2–6.0 \times 10^4$ receptors/cell ensures phosphorylation of the major substrates, full mitogenic response, and good survival, but produces only a weak transforming activity (measured by growth in soft agar). Activation of more than 1×10^5 IGF-IRs provides signal strong enough to activate both IRS-1 and SHC signaling pathways, stimulate cell division, and support robust transformation (16,17). A direct relationship between the number of stimulated IGF-IRs and cell survival and/or tumorigenesis in animal models has been documented by the Baserga and LeRoith laboratories (18,19). For instance, NIH 3T3 mouse fibroblasts expressing 1.9×10^5 IGF-IR/cell form tumors in nude mice, while fibroblasts with lower IGF-IR levels (1.6×10^4) are nontumorigenic. In addition, the latency of tumor formation *in vivo* was reduced with high doses (4–10 mg/kg) of endocrine IGF-I, suggesting that chronic stimulation of a high number of IGF-IRs was critical for the onset of tumorigenesis (19).

A mutational analysis has been performed to determine whether various functions of the IGF-IR are induced by overlapping or distinct pathways. In the studies of Baserga *et al.*, different mutant IGF-IRs have been expressed in R-minus cells (derived from IGF-IR knock-out mice), which allowed the analysis of signaling pathways of the mutants without interference from the endogenous wild-type IGF-IRs (10). This work was complemented by O'Connor *et al.* who studied mutant receptors expressed in either hematopoietic IRS-1-negative FL5.12 cells or apoptosis-prone Rat-1/Myc fibroblasts, and by LeRoith *et al.* who used NIH 3T3 fibroblasts for the analysis (5,6). All studies demonstrated that a mutation in the ATP binding site produced "dead" receptors incapable of signal transmission. Replacement of all three Tyr 1131, 1135, and 1136, or Tyr 1136 alone, with phenylalanine produced a receptor that was not mitogenic or transforming, but it still induced an efficient survival signal. Mutations in either Tyr 1131 or Tyr 1135 downregulated transformation without reducing cell growth. Tyr 950 in the IGF-IR juxtamembrane domain was found necessary for IRS and SHC association, and for induction of mitogenic and transforming activity. Interestingly, however, the IGF-IR/Tyr 950 mutant transmitted anti-apoptotic signaling. This finding indicates that in addition to the classic IRS-1-dependent PI-3K/Akt pathway, other survival pathway(s) emanate from the IGF-IR (5,6,9,10).

Deletion of the entire C-terminus at aa 1229 produced a receptor that retained normal mitogenic function but was totally lacking transforming potential (20). Subsequent detailed studies with mutant IGF-IRs expressed in R-minus cells mapped the "transforming domain" between residues 1245 and 1310, with Tyr 1251, Ser 1280–1283, His 1293, and Lys 1294 required for transformation (10). Importantly, this region does not have an exact counterpart in the IR. Indeed, the expression of the IR or a chimeric IGF-IR containing an IR C-terminus did not support soft agar growth of R-minus cells (10). Notably, the IGF-IR transforming signal appears to be truly unique, at least in mouse fibroblasts, as overexpression of various growth factor receptors, signaling molecules or oncogenes (except for v-src), singly or in combination, did not restore transformation in R-minus cells, while the IGF-IR did (3,21). The mediators of the IGF-IR transforming pathway are not yet known, but the adapters 14-3-3 and

GRB10, which bind to the C-terminus, could be involved.

Interestingly, the IGF-IR C-terminus also appears to play a unique role in survival signaling. Mutants with a deleted C-terminus (at residues 1229 or 1245) retained or even amplified anti-apoptotic function, while single mutations in Tyr 1251, His 1293, and Lys 1294 reduced survival (5). Consequently, it has been suggested that the C-terminus is an intrinsic inhibitory domain of the IGF-IR, while the residues Tyr 1251, His 1293, Lys 1294 act as neutralizers of this pro-apoptotic function. Indeed, expression of the C-terminal 108 aa as a membrane-targeted protein resulted in induction of apoptosis, and mutations in Tyr 1250/1251 and His 1293/Lys 1294 abrogated this cytotoxic activity (5).

To summarize, IGF-IR signals required for mitogenesis, transformation, and survival are distinct but partially overlap. For instance, no transforming activity is seen in the absence of mitogenic activity. Transformation also seems to have some common pathways with IGF-dependent survival. However, cell survival can be induced by a weak signal which is not sufficient for mitogenesis or transformation, while transformation requires strong IGF-IR activation and induction of specific signals originating at the C-terminus.

The pathways mediating non-growth IGF-I responses such as cell-cell or cell-substrate interactions are less well characterized. Our preliminary data indicate that in epithelial cells, intercellular adhesion requires the tyrosine kinase domain as well as the C-terminus of the IGF-IR, and depends on SHC but not on IRS-1 signaling (4,22, Surmacz *et al.*, unpublished data). IGF-I-induced motility and reorganization of actin cytoskeleton involves PI-3K and SHC activities, and modification of proteins associated with focal adhesions (22,23).

It is known that the IGF-IR response may be cell-type specific (24). One mechanism ensuring such specificity is the availability of intracellular signaling intermediates. For instance, with the same cellular content of the IGF-IR, downregulation of IRS-1 expression inhibits cell growth, transformation, and results in cell death, while amplification of IRS-1 sensitizes cells to low concentrations of IGF-I and enhances anchorage-independent growth (22,25,26). On the other hand, overexpression of SHC does not improve IGF-I-dependent growth, but inhibition of SHC expression inhibits cell growth, transformation, and to a lesser extent, cell survival (22,27).

Finally, the extracellular context plays a role in IGF-I response, for instance, survival and growth of cells adhering to a proper substrate is mediated through the IRS-1 pathway, while the same pathway is much less important in IGF-I-dependent protection from apoptosis due to anchorage-independence (22,28,29).

Requirement for IGF-IR in Proliferation, Transformation, and Survival of Breast Cancer Cells

The critical role of the IGF-IR in breast cancer growth, survival, and transformation has been well documented *in vitro* and in animal models (Table I) (4). Reducing ligand availability by excess IGF-BP1 or exposure to suramin blocked IGF-IR activation and limited breast cancer cell proliferation. Furthermore, inhibiting the expression of the IGF-IR with an antisense-IGF-IR RNA, or its function with anti-IGF-IR antibodies or dominant-negative mutants, resulted in growth inhibition and reduced transforming potential (4). Our studies with MCF-7 breast cancer cell lines expressing antisense-IRS-1 or antisense-SHC RNAs (MCF-7/anti-IRS-1 or MCF-7/anti-SHC cells) demonstrated that both IRS-1- and SHC-dependent signals are necessary for cell proliferation and transformation (22). The critical role of IRS-1 (but not IRS-2) and IRS-1 downstream pathways—Ras/MAP and PI-3K in the growth of estrogen receptor (ER)-positive breast cancer cells has recently been confirmed by the Yee laboratory (30). Using dominant-negative IGF-IRs lacking the C-terminus, we demonstrated that in breast tumor cells, as in fibroblasts, the C-terminal portion is essential for transformation *in vitro* and tumorigenesis *in vivo* (4).

Dunn *et al.* have shown that activation of the IGF-IR protects breast cancer cells from apoptosis induced by various therapeutic agents, serum deprivation and irradiation (31). Our results with MCF-7 cells in which IRS-1 has been downregulated by either antisense-IRS-1 oligonucleotides, expression of antisense-IRS-1 RNA, or antiestrogen treatment suggest that the IRS-1/PI-3K signal is required for IGF-IR-induced survival (22,25,32).

Amplification of IGF-IR Signaling and Anchorage-Dependent and -Independent Growth of Breast Cancer Cells

Further understanding of IGF-IR function in breast cancer pathobiology stemmed from studies of

Table I. IGF-IR Function in Breast Cancer

Signaling molecule	Function in breast cancer	
	Experimental models	Tumors
IGF-IR	elevated in ER-positive breast cancer cells; stimulates proliferation; counteracts apoptotic effects of anti-tumor drugs; improves 3-D growth and survival; regulates cell-substrate connections; required for anchorage-independent growth *in vitro* and tumorigenesis and metastasis in animal models.	correlates with the ER status; elevated in primary tumors; high levels correlate with radio-resistance and recurrence at the primary site; usually co-expressed with markers of better overall prognosis.
IRS-1	elevated in ER-positive breast cancer cells; required for anchorage-dependent and independent growth; critical for survival; high levels induce estrogen-independence and antiestrogen-resistance.	correlates with shorter DFS in ER-positive primary tumors.
SHC	required for proliferation, anchorage-independent growth, migration, and cell-cell adhesion; high levels improve adhesion to fibronectin.	?

cells with amplified IGF-IR signaling. In order to correlate the strength of the IGF signal with the progression towards a more neoplastic phenotype, we developed a series of MCF-7-derived cell lines overexpressing different levels of either the IGF-IR (MCF-7/IGF-IR cells), IRS-1 (MCF-7/IRS-1 cells), or SHC (MCF-7/SHC cells) (25,27,29).

Overexpression of the IGF-IR (8–50-fold) was paralleled by enhanced IGF-IR tyrosine kinase activity and hyperphosphorylation of IRS-1, even in the absence of exogenous IGF-I. Compared to the parental cells, all MCF-7/IGF-IR clones exhibited enhanced autocrine growth in serum-free medium and improved growth responsiveness to low concentrations of IGF (0.1–1.0 ng/ml), especially in the presence of 10 nM estradiol (E2). With higher doses of IGF-I (4–50 ng/ml), the synergistic effect was not seen and the maximal mitogenic effect was achieved with IGF-I alone (29). Similar results were described by Daws *et al.*, who independently developed IGF-IR overexpressing MCF-7 clones (33). Interestingly, we as well as others noticed that high doses of IGF-I (20–50 ng/ml) combined with 10 nM E2 inhibited MCF-7/IGF-IR cell growth, especially in the clones with the highest IGF-IR levels (29,33).

Anchorage-independent growth of MCF-7/IGF-IR cells treated with E2 was slightly elevated relative to the parental cells, but this effect of IGF-IR overexpression was not present in cells treated with both E2 and IGF-I or cultured in serum-containing medium (29,33).

In contrast with the modest effects of amplified IGF-IR, overexpression of IRS-1 (1.5–9-fold) produced marked changes in the growth phenotype (25). In MCF-7/IRS-1 cells, proliferation was enhanced under all conditions studied (serum-free and serum-containing medium, or serum-free medium with 20 ng/ml IGF); the addition of E2 never inhibited the growth. Also, MCF-7/IRS-1 cells exhibited greatly a enhanced potential for growth in soft agar, especially in the presence of high (200–400 ng/ml) doses of IGF-I. Remarkably, this IGF-dependent transformation was further potentiated with E2. The above effects were correlated with the cellular levels of IRS-1 and the extent of IRS-1 tyrosine phosphorylation (25).

Amplification of SHC in MCF-7 cells (two–sevenfold) did not alter growth properties under standard monolayer or anchorage-independent conditions, but it amplified cell-substrate interactions on fibronectin (27).

IGF-IR/ER Cross-Talk

In hormone-dependent breast cancer cells, ER and the IGF-IR are co-expressed and E2 acts in synergy with IGF-I to stimulate proliferation (4). The effects of E2 are mediated in part via sensitization of cells to IGF action. E2 treatment up-regulates IGF-IR mRNA and protein levels by two–tenfold, reflected in enhanced IGF-IR tyrosine phosphorylation (4,34,35). Furthermore, E2 significantly (two–fivefold) stimulates the expression of IRS-1 in different ER-positive cell lines, and the extent of this stimulation depends on the cellular ER content (35, Surmacz *et al.*, unpublished data). Of note, E2 action appears to be at least partially specific to the IGF-IR/IRS-1 pathway since it does not modulate SHC levels (36).

Importantly, various antiestrogens such as Tamoxifen (Tam) and its derivatives, droloxifene, and

pure antiestrogens ICI 164,384 and ICI 182,780 inhibit IGF-IR-dependent proliferation (4,32,36–38). We demonstrated that at the molecular level, the anti-IGF-IR actions of Tam and ICI 182,780 are accomplished by downregulation of IRS-1/PI-3 kinase signaling (32,36). Specifically, growth arrest and apoptosis resulting from antiestrogen treatment were associated with continuos suppression of IRS-1 mRNA and protein expression, reflected by reduced IRS-1 tyrosine phosphorylation, decreased IRS-1/PI-3K binding and reduced PI-3K activity (32,36). These anti-IRS-1 effects of ER antagonists were partially reversed in the presence of IGF-I (36).

Antiestrogens also inhibit IGF-IR expression and tyrosine phosphorylation (by 30–50%) but only in the presence of IGF-I (32,36). In the absence of IGF-I, Tam and ICI 182,780 enhance IGF-IR phosphorylation, which suggest that the drugs may act through modulation of IGF-I-dependent phosphatases. Indeed, the involvement of tyrosine phosphatases LAR and FAP-1 in antiestrogen inhibition of IGF-dependent growth has been demonstrated by the Vignon laboratory (39). Interestingly, in different antiestrogen-treated cell lines, SHC expression or signaling were not altered, while SHC tyrosine phosphorylation was increased in Tam- but not in ICI 182,780-arrested cells (32,36).

Because E2 upregulates IGF-IR signaling, it has been postulated that amplification of the IGF-IR or its key signaling substrates may lead to estrogen-independence. In agreement with this hypothesis, MCF-7/IRS-1 cells exhibited reduced estrogen requirements for growth and transformation, and were not inhibited by E2 alone or in combination with of IGF-I (25). Interestingly, however, such estrogen-independence has not been detected in MCF-7/IGF-IR cells (29,33). These cells still appear to remain under ER control as their growth is restrained by high doses of E2 in the presence or absence of IGF-I. This finding suggests the existence of a negative growth regulatory loop which is not operative in MCF-7/IRS-1 cells and may be triggered by hyperactivation of IGF-I signaling pathways not involving IRS-1.

The role of amplified IGF-IR signaling in the development of antiestrogen-resistance is of particular interest. We and others have shown that overexpression of different IGF-IR signaling elements did not affect ER content (25,29,33). However, MCF-7/IRS-1 clones with very high IRS-1 levels (9 or 12-fold overexpression with respect to MCF-7 cells) exhibited resistance to ICI 182,780, confirming that the IRS-1 pathway is an essential target for antiestrogens and suggesting that overexpression of IRS-1 in tumors may hinder antiestrogen therapy (36). Interestingly, in contrast with IRS-1, overexpressed IGF-IRs (50-fold) or SHC (fivefold) did not alter antiestrogen sensitivity in MCF-7 cells (36).

IGF-IR-DEPENDENT CELL-CELL AND CELL-SUBSTRATE INTERACTIONS IN BREAST CANCER CELLS

Breast cancer cells, like other polarized epithelial cells, are governed by cell-cell and cell-substrate interactions. The regulation of these processes by growth factors is now being increasingly recognized. We studied intercellular interactions of MCF-7 cells and their derivatives with modified IGF-IR signaling. We found that overexpression of the IGF-IR greatly enhanced aggregation of cells in three-dimensional (3-D) culture (29). Specifically, when plated on Matrigel, MCF-7/IGF-IR cells formed large spheroids (150–300 μm in diameter) surviving or even proliferating for up to 20 days, while the parental MCF-7 cells formed smaller clusters (50 μm) which disaggregated and died after 7 days of culture. Similar stimulation of cell-cell adhesion has been described in IGF-I treated MCF-7 and MCF-7/6 cells as well as in MCF-7 cells constitutively secreting IGF-I (4). Our subsequent research demonstrated that enhanced cell-cell adhesion is IGF-I-specific as it cannot be induced by physiologic concentrations of EGF, IGF-II or insulin (4). The mechanism of this phenomenon is still not clear. We have shown that the IGF-IR co-localizes with an adherens junction protein E-cadherin and co-precipitates with E-cadherin, α-catenin, and β-catenin (4,29). In addition, we obtained preliminary data suggesting that the IGF-IR stimulates the expression of a junction protein ZO-1, thereby strenghtening the α-catenin/ZO-1/F-actin connections (Surmacz *et al.*, in preparation). The signals required for cell-cell adhesion depend on IGF-IR tyrosine kinase activity and the presence of the C-terminus (4, Surmacz *et al.*, in preparation). We also observed that cell-cell adhesion is reduced in MCF-7/anti-SHC, but not MCF-7/anti-IRS-1 cells, which points to SHC as a putative mediator of IGF-induced aggregation (22).

The enhanced intercellular connections and improved survival of IGF-IR overexpressing cells may contribute to their tumorigenic activity *in vivo*. Indeed, when MCF-7/IGF-IR cells were injected into mam-

mary fat pad of nude mice, they formed tumors after 8 weeks, while the parental MCF-7 cells or MCF-7 clones expressing an IGF-IR with a C-terminal truncation were non-tumorigenic (Surmacz *et al.*, in preparation).

Cell-substrate adhesion and migration of epithelial cells is also regulated by IGF-I. For instance, depolarization of MCF-7 and MCF-7/IGF-IR cells and induction of cell migration can be achieved with a 4 hour treatment with 50 ng/ml IGF-I. The initial stages of this process are associated with transient dephosphorylation of the focal adhesion proteins FAK, paxillin and p130 Cas (23). IGF-IR pathways involved in the regulation of breast cancer cell motility are still quite obscure, but we observed reduced migration of MCF-7 cells with impaired SHC or PI-3K signaling (22,23).

Obviously, extracellular cell context may dictate whether cellular response to IGF-I involves increased cell-cell adhesion or enhanced migration. Increased intercellular adhesion may be seen in cells expressing low levels of integrins necessary for attachment to a given ECM substrate, whereas enhanced migration occurs when the cells interact well with a substrate, or produce sufficient amounts of their own ECM (23,28).

IGF-IR Signaling in Breast Tumors

Studies on IGF-IR expression in breast tumors and its correlation with other host or tumor parameters are very limited (4). Moreover, the interpretation of the available data is complicated by the fact that different techniques were used to assess the IGF-IR levels. The most frequently performed IGF-I binding assay is inherently inaccurate due to the interaction of IGF with membrane IGFBPs, often resulting in overestimation of the number of the IGF-IR (4). To circumvent this problem, the expression of IGF-IR in tumor samples was examined with anti-IGF-IR antibody-based techniques (radioimmunoassay or immunocytochemistry) (40,41). Despite the differences in experimental approach, in all large series studies (>100 cases) the IGF-IR has been detected in a majority of breast tumor samples (4,42,43).

Most important, IGF-IR levels have been found to be elevated (up to 14-fold) in primary breast cancer compared to non-malignant tumors or normal epithelium (40–43) (Table I). The mechanism of the common IGF-IR overexpression in breast cancer is not clear, but it does not appear to be associated with IGF-IR gene amplification since this event was reported in only 2% of cases analyzed (44). Recent data indicate that IGF-IR overexpression may be related to derepression of IGF-IR transcription due to aberrant expression of the tumor supressor protein p53 (40,45,46). Overexpression of the IGF-IR in tumors has been found to be associated with hyperactivation of the tyrosine kinase (up to sixfold), and correlated with radio-resistance and tumor recurrence at the primary site (40,41). High IGF-IR levels in primary tumors have been reported as predictors of shorter disease-free survival, but data on the prognostic value of the IGF-IR for overall survival are conflicting (4,41).

Importantly, not only the IGF-IR, but also IRS-1 has been found to be overexpressed in a fraction of primary breast tumors (35,47). High levels of IRS-1 correlated with shorter disease-free survival in ER-positive tumors (35). The mechanism of IRS-1 overexpression is not known, but it could be associated with E2 or IGF activity since both mitogens are known to stimulate IRS-1 transcription and both can be found (often at superphysiological concentrations) in breast tumors.

Attempts to correlate IGF-IR expression with other host or tumor variables showed a positive link between the IGF-IR and ER status (4,42,43). In addition, frequent co-expression of the IGF-IR and IR has been shown (42). Co-expression of these structurally homologous receptors leads to the formation of functional hybrids which bind IGF-I with high affinity, and thereby amplify the IGF-I signal (48).

In several large series analyses, no significant correlations were found between IGF-IR expression and menopausal status (42), body weight (42), tumor size (42,43), tumor grade (42,49,50), histological type (42,43), node status (42,43,49,50), or EGFR status (49), and the link with the progesterone receptor (PgR) status is uncertain (4,42, 43,49–51). However, because most of these associations were established based on IGF-I binding assays, they should be re-assessed using more accurate techniques of IGF-IR measurement before any firm conclusions can be drawn.

The expression of IRS-1 correlated with ER levels but not with other parameters such as age, tumor size, or PgR status (35,47). The levels of another IGF-IR substrate, SHC, are similar in aggressive and more differentiated breast cancer cell lines, but its activity (tyrosine phosphorylation) in cell lines and tumors reflects the levels of oncogenic kinases ERB2 or c-src

(27,52,53). SHC association with IGF-IR in breast tumors has not been studied.

The Unclear Role of the IGF-IR in Breast Cancer Progression

The experimental and clinical evidence points to the fact that the IGF-IR may be important in early steps of tumor development, promoting cell growth, survival, and resistance to therapeutic treatments. However, the function of the IGF-IR in the later stages, including metastasis, is still obscure.

Especially intriguing is the fact that, whereas the IGF-IR has been found to be overexpressed in primary tumors, its levels, like ER levels, appear to undergo reduction during the course of the disease. For instance, Pezzino *et al.* assessed IGF-IR status in two patient subgroups, representing either a low risk (ER- and PgR-positive, low mitotic index, diploid) or a high risk (ER- and PgR-negative, high mitotic index, aneuploid) population and found a highly significant correlation between IGF-IR expression and better prognosis (42). Similar conclusions were reached by Peyratt and Bonneterre (43). Therefore, it has been proposed that like the ER, the IGF-IR marks more differentiated tumors with better clinical outcome. However, it has also been argued that the IGF-IR may play a role in early steps of tumor spread since node-positive/IGF-IR-positive tumors appeared to have a worse prognosis than node-negative/IGF-IR-positive tumors (49). In addition, quite rare cases of ER-negative but IGF-IR-positive tumors are associated with shorter disease-free survival (48).

In breast cancer cell lines, a hormone-dependent and less aggressive phenotype correlates with a good expression of the IGF-IR and IRS-1 (29,35). In contrast, highly metastatic ER-negative breast cancer cell lines express low levels of the IGF-IR and often do not respond to IGF-I with growth (54,55). Similarly, IRS-1 levels are downregulated in a majority of these cell lines (35,55). Despite this "IGF-IR-reduced phenotype", metastatic cell lines appear to depend on the IGF-IR. For instance, blockade of the IGF-IR in MDA-MB-231 cells by anti-IGF-IR antibody reduced migration *in vitro* and tumorigenesis *in vivo*, and expression of a soluble IGF-IR in MDA-MB-435 cells impaired growth, tumorigenesis and metastasis in animal models (56–58). Whether this particular IGF-I-dependence of metastatic breast cancer cells relates to the survival function of the IGF-IR is under investigation in our laboratory.

CONCLUSIONS

Over the past few years much has been learned about the function of the IGF-IR in the process of tumorigenesis. Clearly, IGF-IR-mediated survival and transformation are key factors affecting tumor development. In primary breast cancer, high levels of the IGF-IR may promote survival and proliferation, counteracting cytotoxic or cytostatic effects of drugs or radiation. The mechanism of this IGF-I action includes strengthening intercellular connections, amplification of anti-apoptotic signals, and sensitization of cells to low concentrations of IGFs and E2. Therefore, targeting the IGF-IR, especially the IGF-IR/IRS-1 pathway, should help in eradicating primary tumor cells.

The importance of the IGF-IR in metastatic breast disease is still not clear. It is possible that the IGF-IR has a role in cell spread, functioning primarily as an anti-apoptotic, and possibly a motogenic factor. Unquestionably, further understanding of IGF-IR function in metastatic cells will be critical in creating successful anti-IGF-IR therapies for late stages of breast cancer.

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Differential Insulin-like Growth Factor I Receptor Signaling and Function in Estrogen Receptor (ER)-positive MCF-7 and ER-negative MDA-MB-231 Breast Cancer Cells[1]

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ABSTRACT

The insulin-like growth factor I receptor (IGF-IR) is a ubiquitous and multifunctional tyrosine kinase that has been implicated in breast cancer development. In estrogen receptor (ER)-positive breast tumors, the levels of the IGF-IR and its substrate, insulin-receptor substrate 1 (IRS-1), are often elevated, and these characteristics have been linked with increased radioresistance and cancer recurrence. *In vitro*, activation of the IGF-IR/IRS-1 pathway in ER-positive cells improves growth and counteracts apoptosis induced by anticancer treatments. The function of the IGF-IR in hormone-independent breast cancer is not clear. ER-negative breast cancer cells often express low levels of the IGF-IR and fail to respond to IGF-I with mitogenesis. On the other hand, anti-IGF-IR strategies effectively reduced metastatic potential of different ER-negative cell lines, suggesting a role of this receptor in late stages of the disease.

Here we examined IGF-IR signaling and function in ER-negative MDA-MB-231 breast cancer cells and their IGF-IR-overexpressing derivatives. We demonstrated that IGF-I acts as a chemoattractant for these cells. The extent of IGF-I-induced migration reflected IGF-IR levels and required the activation of phosphatidylinositol 3-kinase (PI-3K) and p38 kinases. The same pathways promoted IGF-I-dependent motility in ER-positive MCF-7 cells. In contrast with the positive effects on cell migration, IGF-I was unable to stimulate growth or improve survival in MDA-MB-231 cells, whereas it induced mitogenic and antiapoptotic effects in MCF-7 cells. Moreover, IGF-I partially restored growth in ER-positive cells treated with PI-3K and ERK1/ERK2 inhibitors, whereas it had no protective effects in ER-negative cells. The impaired IGF-I growth response of ER-negative cells was not caused by a low IGF-IR expression, defective IGF-IR tyrosine phosphorylation, or improper tyrosine phosphorylation of IRS-1. Also, the acute (15-min) IGF-I activation of PI-3 and Akt kinases was similar in ER-negative and ER-positive cells. However, a chronic (2-day) IGF-I exposure induced the PI-3K/Akt pathway only in MCF-7 cells. The reactivation of this pathway in ER-negative cells by overexpression of constitutively active Akt mutants was not sufficient to significantly improve proliferation or survival (with or without IGF-I), which indicated that other pathways are also required to support these functions.

Our results suggest that in breast cancer cells, IGF-IR can control nonmitogenic processes regardless of the ER status, whereas IGF-IR growth-related functions may depend on ER expression.

INTRODUCTION

The IGF-IR[3] is a ubiquitous, transmembrane tyrosine kinase that has been implicated in different growth-related and growth-unrelated processes critical for the development and progression of malignant tumors, such as proliferation, survival, and anchorage-independent growth, as well as cell adhesion, migration, and invasion (1, 2).

The IGF-IR is necessary for normal breast biology, but recent clinical and experimental data strongly suggest that the same receptor is involved in the development of breast cancer (1, 3). The IGF-IR is overexpressed (up to 14-fold) in ER-positive breast cancer cells compared with its levels in normal epithelial cells (1, 4, 5). The elevated expression and hyperactivation of the IGF-IR has been linked with increased radioresistance and cancer recurrence at the primary site (4). Similarly, high levels of IRS-1, a major signaling molecule of the IGF-IR, correlated with tumor size and shorter disease-free survival in ER-positive tumors (6, 7).

IGF-IR ligands, IGF-I and IGF-II, are strong mitogens for many hormone-dependent breast cancer cell lines and have been found in the epithelial and/or stromal component of breast tumors (1). Importantly, higher levels of circulating IGF-I predict increased breast cancer risk in premenopausal women (8). *In vitro*, activation of the IGF-IR, especially the IGF-IR/IRS-1/PI-3K pathway in ER-positive breast cancer cells, counteracts apoptosis induced by different anticancer treatments or low concentrations of hormones (1, 9–11). On the other hand, overexpression of either the IGF-IR or IRS-1 in ER-positive breast cancer cells improves responsiveness to IGF and, in consequence, results in estrogen-independent proliferation (1, 12, 13). In agreement with these observations, blockade of IGF-IR activity with various reagents targeting the IGF-IR or its signaling through IRS-1/PI-3K reduced the growth of breast cancer cells *in vitro* and/or *in vivo* (1, 12, 14–17).

The requirement for the IGF-IR/IRS-1 pathway for growth and survival appears to be a characteristic of ER-positive, more differentiated, breast cancer cells. By contrast, ER-negative tumors and cell lines, often exhibiting less differentiated, mesenchymal phenotypes, express low levels of the IGF-IR and often decreased levels of IRS-1 (1, 17, 18). Notably, these cells do not respond to IGF-I with growth (1, 19–22). Despite the lack of IGF-I mitogenic response, the metastatic potential of ER-negative breast cancer cells can be effectively inhibited by different compounds targeting the IGF-IR. For instance, blockade of the IGF-IR in MDA-MB-231 cells by an anti-IGF-IR antibody reduced migration *in vitro* and tumorigenesis *in vivo*, and expression of a soluble IGF-IR in MDA-MB-435 cells inhibited adhesion on the extracellular matrix and impaired metastasis in animals (14, 16, 23). These observations suggested that in ER-negative cells, some functions of the IGF-IR must be critical for metastatic cell spread. Here we addressed the possibility that in ER-negative cells, the IGF-IR selectively promotes growth-unrelated processes, such as migration and invasion, but is not engaged in the transmission of growth and survival signals. Using ER-negative MDA-MB-231 breast cancer cells, we studied IGF-I-dependent pathways involved in migration and the defects in IGF mitogenic signal. For comparison, relevant IGF-I responses were analyzed in ER-positive MCF-7 cells.

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[3] The abbreviations used are: IGF-IR, insulin-like growth factor I receptor; IRS-1, insulin-receptor substrate 1; ER, estrogen receptor; GFP, green fluorescent protein; mAb, monoclonal antibody; WB, Western blot; CS, calf serum; IP, immunoprecipitation; pAb, polyclonal antibody; MAPK, mitogen-activated protein kinase; PI-3K, phosphatidylinositol 3-kinase; ERK, extracellular signal-regulated kinase; GSK, glycogen synthase kinase; MEK, MAPK kinase; PRF-SFM, phenol red-free serum-free medium.

MATERIALS AND METHODS

Plasmids. The pcDNA3-IGF-IR expression plasmid encoding the wild-type IGF-IR under the cytomegalovirus promoter was described before (13). The expression plasmids encoding constitutively active forms of Akt kinase, *i.e.*, myristylated Akt and Akt with an activating point mutation (Akt/E40K), were obtained from Drs. P. Tsichlis and T. Chan (Kimmel Cancer Center) and were described before (24). The Akt plasmids contain the HA-tag, allowing for easy identification of Akt-transfected cells. The pCMS-EGFP expression vector encoding GFP was purchased from Clontech.

Cell Lines. MDA-MB-231 cells were obtained from American Type Culture Collection. MDA-MB-231/IGF-IR clones were generated by stable transfection of MDA-MB-231 cells with the plasmid pcDNA3-IGF-IR using a standard calcium phosphate precipitate procedure (13). Transfectants resistant to 1 mg/ml G418 were screened for IGF-IR expression by fluorescence-assisted cell sorting analysis using an anti-IGF-IR mouse mAb α-IR3 (10 μg/ml; Calbiochem) and a fluorescein-conjugated goat antimouse IgG2 (1 μg/ml; Calbiochem). Cells stained with the secondary antibody alone were used as a control. Additionally, the parental MDA-MB-231 cells and MCF-7/IGF-IR clones 12 and 15 (13), all expressing known levels of the IGF-IR, were analyzed in parallel. IGF-IR expression in MDA-MB-213-derived clones was then confirmed by WB with specific antibodies (listed below). In growth and migration experiments, we used control MCF-7/pc2 and MDA-MB-231/5 M cell lines, which have been developed by transfection of MCF-7 and MDA-MB-231 cells with the pcDNA3 vector. MCF-7, MCF-7/pc2, and MCF-7/IGF-IR cells were described in detail previously (13).

Transient Transfection. Seventy % confluent cultures of MDA-MB-231 and MCF-7 cells were transiently cotransfected with an Akt expression plasmid and a plasmid pCMS encoding GFP (Akt:GFP ratio, 20:1) using Fugene 6 (Roche). Transfection was carried out for 6 h in phenol red-free DMEM containing 0.5 mg/ml BSA, 1 μM $FeSO_4$, and 2 mM L-glutamine (referred to as PRF-SFM; Ref. 13); the optimal DNA:Fugene 6 ratio was 1 μg:3 μl. Upon transfection, the cells were shifted to fresh PRF-SFM, and the expression of total and active Akt kinase at 0 (media shift), 2, and 4 days was assessed by WB with specific antibodies (see below). In parallel, the efficiency of transfection was evaluated by scoring GFP-positive cells. In all experiments, at least 40% of transfected cells expressed GFP, which indicated a high transfection efficiency. In addition, the expression of Akt plasmids was monitored by measuring the cellular levels of HA-tag and Akt proteins by WB.

Cell Culture. MDA-MB-231 and MCF-7 cells were grown in DMEM:F12 (1:1) containing 5% CS. MDA-MB-231- and MCF-7-derived clones overexpressing the IGF-IR or expressing vector alone were maintained in DMEM:F12 plus 5% CS plus 200 μg/ml G418. In the experiments requiring 17β-estradiol- and serum-free conditions, the cells were cultured in PRF-SFM (13).

Growth Curves. To analyze the growth in serum-containing medium, the cells were plated in six-well plates in DMEM:F12 (1:1) containing 5% CS at a concentration of 1.5–2.0 $\times$ 10^5 cells/plate; the number of cells was then assessed by direct counting at 1, 2, and 4 days after plating. To study IGF-I-dependent proliferation, the cells were plated in six-well plates in the growth medium as above. The following day (day 0), the cells at ~50% confluence were shifted to PRF-SFM containing 20 ng/ml IGF-I. Cell number was determined at days 1, 2, and 4.

Apoptosis Assay. The cells grown on coverslips in normal growth medium were shifted to PRF-SFM at 70% confluence and then cultured in the presence or absence of 20 ng/ml IGF-I for 0, 12, 24, 48, and 96 h. Apoptosis in the cultures was determined with the *In Situ* Cell Death Detection kit, Fluorescein (Roche), following the manufacturer's instructions. The cells containing DNA strand breaks were stained with fluorescein-dUTP and detected by fluorescence microscopy. Cells that detached during the experiment were spun on glass slides using cytospin and processed as above. Apoptotic index (the percentage of apoptotic cells/total cell number in a sample field) was determined for adherent and floating cell populations, and the indices were combined.

Immunoprecipitation and Western Blotting. Seventy % cultures were shifted to PRF-SFM for 24 h and then stimulated with 20 ng/ml IGF-I for 15 min, 1 h, 1 day, or 2 days. Proteins were obtained by lysing the cells in a buffer composed of 50 mM HEPES (pH 7.5), 150 mM 1% Triton X-100, 1.5 mM $MgCl_2$, 1 mM $CaCl_2$, 5 mM EGTA, 10% glycerin, 0.2 mM Na_3VO_4, 1% phenylmethylsulfonyl fluoride, and 1% aprotinin. The IGF-IR was immunoprecipitated from 500 μg of protein lysate with anti-IGF-IR mAb (Calbiochem) and subsequently detected by WB with anti-IGF-IR pAb (Santa Cruz Biotechnology). IRS-1 was precipitated from 500 μg of lysate with anti-IRS-1 pAb (UBI) and detected by WB using the same antibody. Tyrosine phosphorylation (PY) of immunoprecipitated IRS-1 or IGF-IR was assessed by WB with anti-phosphotyrosine mAb PY20 (Transduction Laboratories). Akt, ERK1/ERK2, and p38 MAPKs (active and total), and active GSK-3 were measured by WB in 50 μg of whole cell lysates with appropriate antibodies from New England Biolabs. The expression of HA-tag was probed by WB in 50 μg of protein lysate with anti-HA mAb (Babco). The intensity of bands representing relevant proteins was measured by laser densitometry scanning.

IRS-1-associated PI-3K Activity. PI-3K activity was determined *in vitro*, as described by us before (25). Briefly, 70% cultures were synchronized in PRF-SFM for 24 h and then stimulated with 20 ng of IGF-I for 15 min or 2 days. Untreated cells were used as controls. IRS-1 was precipitated from 500 μg of cell lysates; IRS-1 IPs were then incubated in the presence of inositol and [^{32}P]ATP for 30 min at room temperature. The products of the kinase reaction were analyzed by TLC using TLC plates (Eastman Kodak). Radioactive spots representing phosphatidylinositol-3-phosphates were visualized by autoradiography, quantified by laser densitometry (ULTRO Scan XL, Pharmacia), and then excised from the plates and counted in a beta counter.

Motility Assay. Chemotaxis and chemokinesis were tested in modified Boyden chambers containing porous (8-mm), polycarbonate membranes. The membranes were not coated with extracellular matrix. Briefly, 2 $\times$ 10^4 cells (synchronized in PRF-SFM for 24 h) were suspended in 200 μl of PRF-SFM and plated into upper wells. Lower wells contained 500 μl of PRF-SFM. To study chemotaxis, IGF-I (20 ng/ml) was added to lower wells only; to assess chemokinesis, IGF-I was placed in either upper wells only, or in both wells. After 24 h, the cells in the upper wells were removed, whereas the cells that migrated to the lower wells were fixed and stained in Coomassie Blue solution (0.25 g of Coomassie blue:45 ml water:45 ml methanol:10 ml glacial acetic acid) for 5 min. After that, the chambers were washed three times with H_2O. The cells that migrated to the lower wells were counted under the microscope (10, 26).

Inhibitors of PI-3K and MAPK. LY294002 (Biomol Research Labs) was used to specifically inhibit PI-3K (27). UO126 (Calbiochem), a specific inhibitor of MEK1/2, was used to block ERK1 and ERK2 kinases (28), and SB203580 (Calbiochem) was used to down-regulate p38 MAPK (29). To determine optimal concentrations of the compounds, different doses (1–100 μM) of the inhibitors were tested in cells treated for 1, 8, 12, and 24 h in PRF-SFM. Additionally, the efficacy of all inhibitors in blocking the phosphorylation of relevant downstream targets (Akt, ERK1/ERK2, and p38 kinases) was determined by WB. In this experiment, the cells were stimulated with IGF-I (20 ng/ml) for 15 min. LY294002 and UO126 were supplemented simultaneously with IGF-I, whereas SB203580 was added 30 min before IGF-I treatment. Ultimately, for both growth and migration experiments, LY294002 was used at the concentration 50 μM, UO126 at 5 μM, and SB203580 at 10 μM. At these doses, the inhibitors did not affect cell proliferation and survival at 24 h, with the exemption of LY294002, which inhibited (by 20%) the proliferation of MCF-7/IGF-IR clone 12 in PRF-SFM. A shorter treatment (12 h) with LY294002 had no impact on the growth and survival of the cells (evaluated by cell proliferation and *In Situ* Cell Death Detection assays, as described above). Thus, the effects of LY294002 on migration were assessed at 12 h, whereas the actions of UO126 and SB203580 were assessed at 24 h of treatment.

RESULTS

MDA-MB-231/IGF-IR Cells. To study growth-related and growth-unrelated effects of IGF-I in ER-negative cells breast cancer cells, we used the MDA-MB-231 cell line. These cells express low levels of the IGF-IR and do not respond to IGF-I with growth (19, 22). Because it has been established that mitogenic response to IGF-I requires a threshold level of the IGF-IR (*e.g.*, in NIH 3T3-like fibroblasts, ~1.5 $\times$ 10^4 IGF-IRs; Refs. 30, 31), our first goal was to test whether increasing IGF-IR expression would induce IGF-I-dependent growth in MDA-MB-231 cells. To this end, several MDA-MB-231 clones overexpressing the IGF-IR (MDA-MB-231/IGF-IR

cells) were generated by stable transfection, and the receptor content was analyzed by binding assay, fluorescence-assisted cell sorting analysis (data not shown), and WB (Fig. 1). We determined that MDA-MB-231 clones 2, 21, and 31 express approximately 3×10^4, 1.5×10^4, and 2.5×10^5 IGF-IRs/cell, respectively, whereas the parental MDA-MB-231 cells express approximately 7×10^3 IGF-IRs/cell (19). For comparison, $\sim 6 \times 10^4$ IGF-IRs were found in ER-positive MCF-7 cells (Fig. 1; Ref. 13).

IGF-IR Overexpression Does Not Enhance the Growth of MDA-MB-231/IGF-IR Cells in Serum-containing Medium. The analysis of growth profiles of different MDA-MB-231/IGF-IR clones indicated that overexpression of the IGF-IR never improved basal proliferation in normal growth medium, and in the case of clone 31, which expressed the highest IGF-IR content ($\sim 2.5 \times 10^5$ IGF-IRs/cell), an evident growth retardation at days 2 and 4 ($P < 0.05$) was observed (Fig. 2*A*). In contrast, similar overexpression of the IGF-IR in ER-positive MCF-7 cells significantly augmented proliferation (Fig. 2*B*). The growth of control clones MDA-MB-231/5 M and MCF-7/pc2 was comparable with that of the corresponding parental cell lines (Fig. 2).

IGF-IR Overexpression Does Not Promote IGF-I-dependent Growth or Survival of MDA-MB-321 Cells. Subsequent studies established that increasing the levels of the IGF-IR from 7×10^3 up to 2.5×10^5 was not sufficient to induce IGF-I-dependent growth response in MDA-MB-231 cells. In fact, similar to the parental and MDA-MB-231/5 M cells, all MDA-MB-231/IGF-IR clones were progressively dying in PRF-SFM with or without 20 ng/ml IGF-I (Fig. 3*A*). In all ER-negative cell lines, the rate of cell death was significantly increased at days 2 and 4 of the experiment. Notably, at these later time points, MDA-MB-231/IGF-IR clone 31 was dying faster in the presence of IGF-I than in PRF-SFM and more rapidly than the parental cells (Fig. 3*A* and data not shown). Conversely, in ER-positive cells, the stimulation of the IGF-IR always induced proliferation. In addition, at later time points, especially at day 4, the growth rate in IGF-I was significantly ($P < 0.05$) increased in MCF-7/IGF-IR cells relative to that in MCF-7 or MCF-7/pc2 cells (Fig. 3*B*).

The analysis of the antiapoptotic effects of IGF-I in the above cell lines cultured for 48 h under PRF-SFM indicated that IGF-I reduced apoptosis, by ~3-fold, in ER-positive cells, but it was totally ineffective in MDA-MB-231 and MDA-MB-231/IGF-IR cells (Table 1).

IGF-IR Signaling in MDA-MB-231 and MDA-MB-231/IGF-IR Cells. Next, we investigated molecular basis underlying the lack of IGF-I growth response in ER-negative cells. IGF-I signaling was studied in MDA-MB-231 cells, MDA-MB-231, clone 31, and in parallel, in ER-positive MCF-7 and MCF-7/IGF-IR cells. The experiments focused on IGF-IR tyrosine kinase activity and several postreceptor signaling pathways that are known to control the growth and survival of ER-positive breast cancer cells (and many other cell types), *i.e.*, the IRS-1/PI-3K, Akt, and ERK1/ERK2 pathways (1, 17, 25, 32–34). We also analyzed other IGF-I effectors that have been

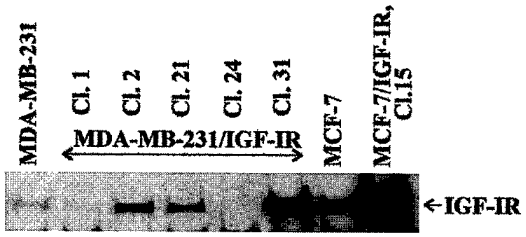

Fig. 1. MDA-MB-231/IGF-IR clones. MDA-MB-231/IGF-IR cells were generated by stable transfection with an IGF-IR expression vector, as described in "Materials and Methods." In several G418-resistant clones, the expression of the IGF-IR protein was tested in 50 μg of total protein lysate by WB with anti-β subunit IGF-IR pAb (Santa Cruz Biotechnology). For comparison, MCF-7 cells and MCF-7/IGF-IR clone 15 with known levels of IGF-IR (6×10^4 and 3×10^6, respectively; Ref. 13) are shown. Low levels of IGF-IR in MDA-MD-231 cells ($\sim 7 \times 10^3$ receptors/cell) are not well visible in this blot but were detectable in its phosphorylated form by IP and WB in 500 μg of protein lysates (see Fig. 4*A*). The estimated expression of the IGF-IR in clones 2, 21, and 31 is 1.5×10^4, 3×10^4, and 2.5×10^5 receptors/cell, respectively.

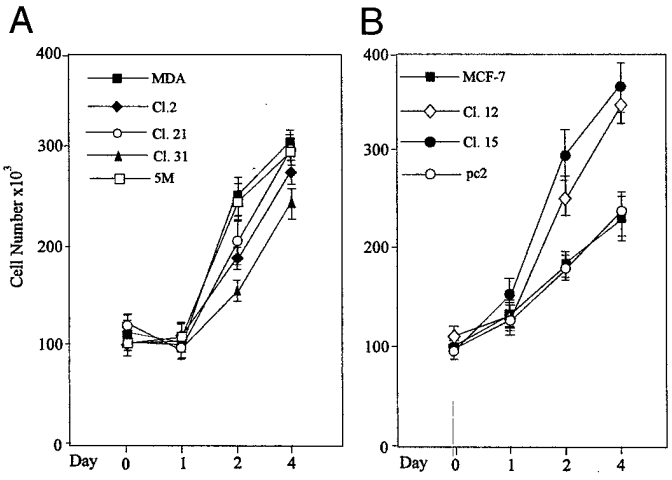

Fig. 2. Effect of IGF-IR overexpression on the growth of ER-negative and ER-positive cells in serum-containing medium. MDA-MB-231 cells, MDA-MB-231/IGF-IR clones 2, 21, and 31 (*A*), and their ER-positive counterparts, MCF-7 cells and MCF-7/IGF-IR, clones ER 12 and 15 (*B*), were plated in DMEM:F12 plus 5% CS. The cells were counted at 50% confluence (day 0) and at subsequent days 1, 2, and 4. Control clones MDA-MB-231/5 M and MCF-7/pc2 expressing the pcDNA3 vector alone were used as controls (*A* and *B*). The results are averages from three experiments. *Bars,* SD.

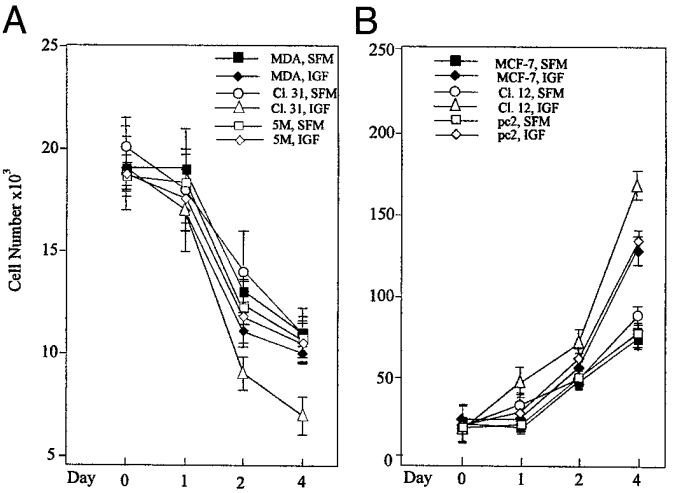

Fig. 3. IGF-I-dependent growth and survival of ER-negative and ER-positive breast cancer cells. ER-negative (*A*) and ER-positive (*B*) cells were synchronized in PRF-SFM and treated with IGF-I, as described in "Materials and Methods." The cells were counted at days 0, 1, 2, and 4 of treatment. The results are averages from at least three experiments. *Bars,* SD.

Table 1 *Effects of IGF-I on apoptosis in ER-negative and ER-positive cells*

Apoptosis was studied in MDA-MB-231 cells, MDA-MB-231/IGF-IR clone 31, MCF-7 cells, and MCF-7/IGF-IR clone 12. The cells were cultured for 48 h in PRF-SFM, and the apoptotic index (% apoptotic cells/total cell number in the field) was determined by terminal deoxynucleotidyl transferase-mediated nick end labeling, as described in "Materials and Methods." The results are averages from at least three experiments; SDs are given.

	Apoptosis (%)	
Cell line	SFM	SFM + IGF-I
MDA-MB-231	41.4 ± 3.0	46.0 ± 1.9
MDA-MB-231/IGF-IR	50.1 ± 4.1	53.3 ± 4.2
MCF-7	14.5 ± 0.2	4.2 ± 0.1
MCF-7/IGF-IR	10.1 ± 1.3	2.8 ± 0.1

shown to contribute to nonmitogenic responses in ER-positive breast cancer cells, such as p38 kinase and SHC (10, 26, 35).

Because both acute and chronic effects of growth factors are important for biological response (36), we studied IGF-IR signaling at different times after stimulation: 15 min, 1 h, 2 days, and 4 days. In both ER-positive and ER-negative cell types, IGF-I signaling seen at 15 min was identical to that at 1 h, whereas IGF-I response at 2 days was similar to that at 4 days. Thus, Fig. 4 demonstrates the representative results obtained with cells stimulated for 15 min and 2 days.

In MDA-MB-231 and MDA-MB-231/IGF-IR cells, IGF-IR and its major substrate, IRS-1, were tyrosine phosphorylated at both time points in a manner roughly reflecting the receptor levels. The activation of both molecules was stronger just after stimulation and weaker at 2 days of the treatment (Fig. 4*A*). Analogous IGF-I effects were seen in MCF-7 cells and their IGF-IR-overexpressing derivatives (Fig. 4*B*). A basal level of IGF-IR and IRS-1 tyrosine phosphorylation was observed in cells expressing high receptor levels. This effect most likely can be attributed to the autocrine stimulation of the IGF-IR by IGF-I-like factors (12).

One of the major growth/survival pathways initiated at IRS-1 is the PI-3K pathway (32, 37). The repeated measurements of IRS-1-associated PI-3K activity *in vitro* demonstrated that at 15 min after IGF-I addition, PI-3K activity was similar in both cell types, but at 2 days, in MDA-MB-231 and MDA-MB-231/IGF-IR cells, IGF-I did not stimulate PI-3K through IRS-1, or induced it very weakly, whereas in MCF-7 and MCF-7/IGF-IR cells, a significant level of PI-3K activation was observed (Fig. 5).

The *in vitro* activity of PI-3K was reflected by the stimulation of its downstream effector, Akt kinase. At 15 min, Akt was up-regulated in response to IGF-I an all cell lines, but at 2 days, no effects of IGF-I were seen in MDA-MB-231 and MDA-MB-231/IGF-IR cells, whereas up-regulation of Akt was still evident in MCF-7 and MCF-7/IGF-IR cells (Fig. 4, *C* and *D*). Akt is known to phosphorylate (on Ser-9) and down-regulate GSK-3β (23, 32, 34). We found that in both cell types, the phosphorylation of GSK-3β reflected the dynamics of Akt activity, with no induction of phosphorylation observed at 2 days in ER-negative cells (Fig. 4*C*) and IGF-I-stimulated phosphorylation in MCF-7 and MCF-7/IGF-IR cells (by 40 and 120%, respectively; Fig. 4*D*).

Another IGF-IR growth/survival pathway involves ERK1 and ERK2 kinases (1, 36, 38). This pathway was strongly up-regulated at 15 min and weakly induced at 2 days in MCF-7 and MCF-7/IGF-IR cells. In MDA-MB-231 and MDA-MB-231/IGF-IR cells, the basal activation of ERK1/2 kinases was always high, and the addition of IGF-I only minimally (10–20%) induced the enzymes at 15 min, with no effects seen at 2 days (Fig. 4, *E* and *F*).

p38, a stress-induced MAPK and a known mediator of nongrowth responses in breast cancer cells (35), was strongly stimulated by IGF-I in ER-negative cells at 15 min (Fig. 4*E*). By contrast, in ER-positive cells, the enzyme was much stronger when induced at 2 days than at 15 min (Fig. 4*F*). The stimulation of SHC, a substrate of the IGF-IR involved in migration and growth in ER-positive cells (10, 26), was weak in all cell types, and no differences in the activation patterns were observed (data not shown).

Reactivation of Akt Kinase in MDA-MB-231 Cells. Previous results indicated that MDA-MB-231 and MDA-MB-231/IGF-IR cells are unable to sustain IGF-I-dependent activation of the PI-3K/Akt survival pathway when cultured in the absence of serum for 2–4 days. Consequently, we tested whether cell death under PRF-SFM conditions can be reversed by a forced overexpression of the Akt kinase. Two different expression plasmids encoding constitutively active forms of Akt, Myr-Akt, and Akt/E40K (24) were transiently transfected into MDA-MB-231 cells. The efficiency of transfection was at least 40% (by scoring GFP-positive cells); correspondingly, the transfected cells expressed elevated (by ~40%) levels of the Akt protein and exhibited enhanced Akt phosphorylation (Fig. 6*A*). The improved biological activity of Akt in the transfected cells was indicated by down-regulation of the prolonged ERK1/2 stimulation (39, 40), which was noticeable at day 2 (data not shown) and most pronounced at day 4 (~50 and 40% for Myr-Akt and Akt/E40K, respectively; Fig. 6*B*) The expression of constitutively active Akt mutants was reflected by a tendency of MDA-MB-231 cells to survive better at 2 days after transfection (at the time of the greatest Akt activity), but the differ-

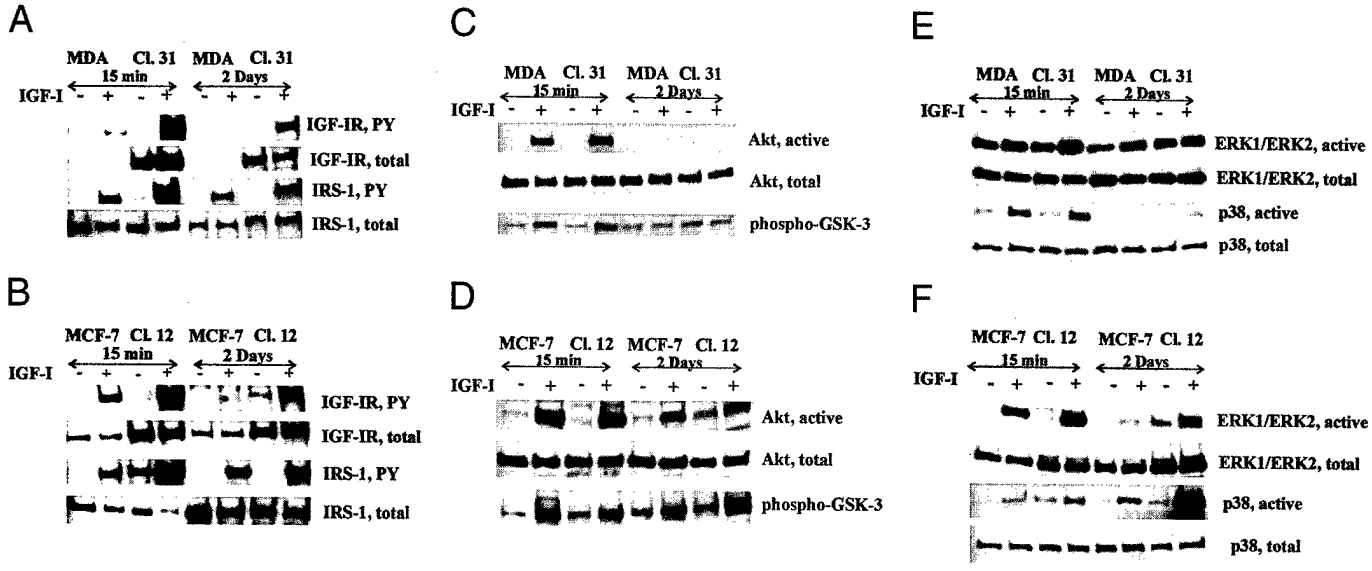

Fig. 4. IGF-I signaling in ER-negative and ER-positive breast cancer cells. The activation of IGF-IR/IRS-1 signaling (*A* and *B*), Akt/GSK-3 signaling (*C* and *D*), and ERK1/ERK2 and p38 kinase signaling (*E* and *F*) was tested in MDA-MB-231 cells, MDA-MB-231/IGF-IR clone 31, MCF-7 cells, and MCF-7/IGF-IR clone 12. The cells were synchronized in PRF-SFM and treated with IGF-I for 15 min or 2 days. The cellular levels of the IGF-IR and IRS-1 were detected by IP and WB in 500 μg of total protein lysate using specific antibodies (see "Materials and Methods"). IGF-IR and IRS-1 tyrosine phosphorylation (PY) was assessed upon stripping and reprobing the same filters with the anti-PY20 antibody. The levels and activity of Akt, GSK-3, ERK1/ERK2, and p38 kinases were probed by WB in 50 μg of total cellular lysates using specific antibodies. The antibodies used are listed in "Materials and Methods." Representative results of at least three repeats are shown. Note decreased IRS-1 expression in *15 min IGF-I* treatment in ER-positive cells, as described before (47).

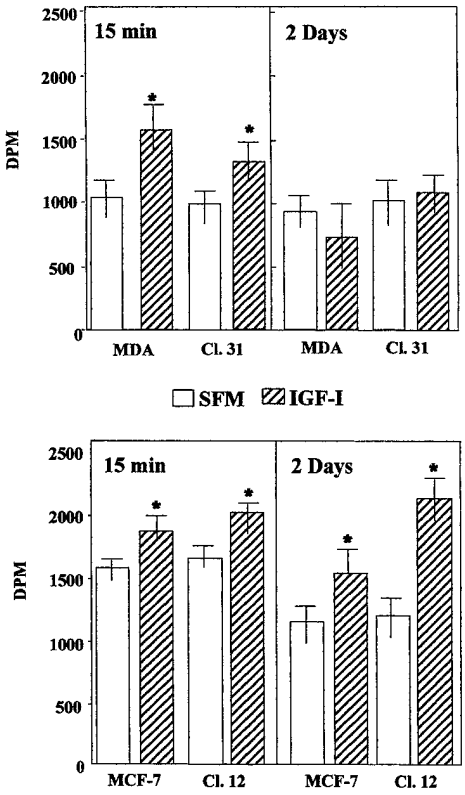

Fig. 5. IGF-I-induced PI-3K activity in ER-negative and ER-positive cells. MDA-MB-231 cells, MDA-MB-231/IGF-IR clone 31, MCF-7 cells, and MCF-7/IGF-IR clone 12 were synchronized in PRF-SFM and treated with IGF-I for 15 min or 2 days. IRS-1-bound PI-3K activity was measured *in vitro* in IRS-1 IPs as described in "Materials and Methods." The experiments were repeated three times for ER-positive cells and five times for ER-negative cells. *Bars*, SD. *, statistically significant differences between untreated and IGF-I-treated cells.

ences did not reach statistical significance ($P > 0.05$; Fig. 6*C* and data not shown).

Inhibition of IGF-IR Signaling Pathways. To complement the above studies, we examined the importance of the PI-3K, ERK1/ERK2, and p38 kinase pathways in IGF-I-dependent growth and survival of ER-positive and ER-negative breast cancer cells using specific inhibitors (27–29). The efficacy of PI-3K and ERK1/ERK2 inhibitors was first tested by establishing their effects on the activity of target proteins (Fig. 7). Table 2 demonstrates the impact of the compounds on cell growth/survival at 2 days of treatment. The inhibition of PI-3K with LY294002 reduced the growth of MCF-7 and MCF-7/IGF-IR cells, but it did not influence or had only minimal effects on MDA-MB-231 and MDA-MB-231/IGF-IR cells. Furthermore, the action of LY294002 was counteracted by IGF-I in ER-positive, but not in ER-negative, cells. The inhibition of MEK1/2 and ERK1/ERK2 with UO126 reduced the growth and/or survival in both cell types, but only in MCF-7 and MCF-7/IGF-IR cells was IGF-I able to oppose this effect. Down-regulation of p38 kinase with SB203580 reduced the survival of MDA-MB-231 and MDA-MB-231/IGF-IR cells and to a lesser extent the growth and survival of MCF-7 and MCF-7/IGF-IR cells. IGF-I did not reverse the antimitogenic action of the p38 kinase inhibitor in either of the cell lines studied (Table 2). Cumulatively, these results suggested that in ER-positive cells, IGF-I transmits mitogenic signals through PI-3K and ERK1/ERK2 pathways. By contrast, IGF-I does not induce growth or survival signal through these pathways in ER-negative cells.

IGF-I Stimulates Migration of MDA-MB-231 Cells. We investigated the nonmitogenic effects of IGF-I in ER-negative and ER-positive breast cancer cells. Unlike with the growth and survival responses, we found that the IGF-IR transmitted nonmitogenic signals in MDA-MB-231 and MDA-MB-231/IGF-IR cells. Specifically, in the chemotaxis experiments, IGF-I placed in lower wells stimulated migration of ER-negative cells in a manner reflecting IGF-IR content. Similarly, the same IGF-I doses induced migration in ER-positive cells (Table 3). The addition of IGF-I to the upper well or both upper and lower wells always suppressed chemotaxis of all cell lines (Table 3).

IGF-I Pathways Regulating Migration of MDA-MB-231 Cells. Using the inhibitors of PI-3K, ERK1/ERK2, and p38 kinases, we determined which pathways of the IGF-IR are involved in migration of ER-negative and ER-positive cells. The treatment was carried out for 24 h (UO126 and SB203580) or 12 h (LY294002) and did not affect cell growth and/or survival with or without IGF-I (see "Materials and Methods"). As demonstrated in Table 4, down-regulation of PI-3K with LY294002 inhibited basal migration of both cell types, with a more pronounced effect in ER-negative cells. Similarly, blockade of p38 kinase inhibited motility of all cell lines studied. The inhibition of MEK1/2 and ERK1/2 with UO126 never suppressed the

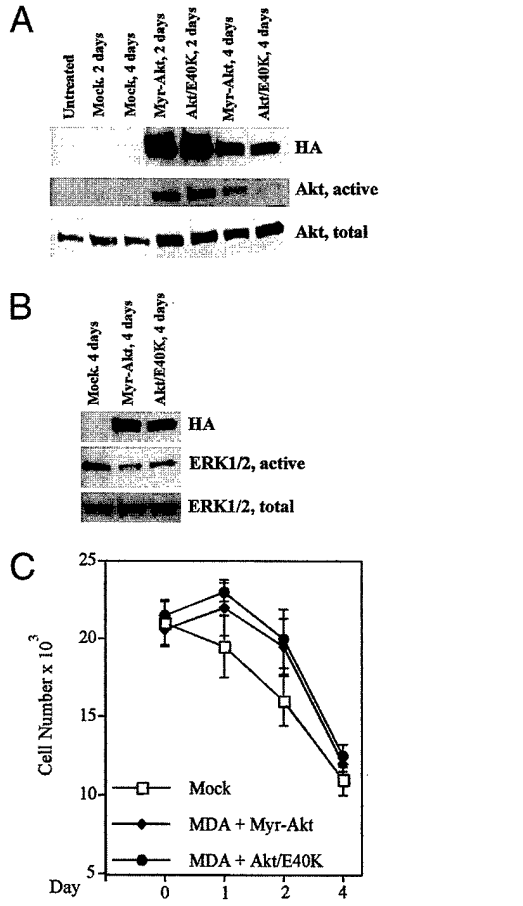

Fig. 6. Effect of increased Akt activity on the survival of MDA-MB-231 cells. MDA-MB-231 cells were transiently transfected with expression plasmids encoding two different constitutively active Akt kinase mutants (Myr-Akt and Akt/E40K; Ref. 24). The Akt vectors contained HA-tag for easy detection. The cells treated with the transfection mixture lacking plasmid DNA (*Mock*) served as control. The expression of the plasmids, as well as the activity and the levels of Akt kinase in the transfected cells, was monitored at 2 and 4 days after transfection. Fifty μg of total protein lysates were sequentially probed by WB with anti-HA, anti-active Akt and then anti-total Akt-specific antibodies (described in "Materials and Methods"). Representative results of four repeats are shown (*A*). To assess biological activity of Akt, the levels of total and active ERK1/2 in cells transfected with the Myr-Akt expression vector were probed in 50 μg of total cell lysate using specific antibodies; the inhibition of ERK1/2 at 4 days after transfection is shown (*B*). *C*, in parallel, the number of cells was determined at days 0 (posttransfection medium change), 1, 2, and 4 after transfection. The results are averages from four experiments. *Bars*, SD.

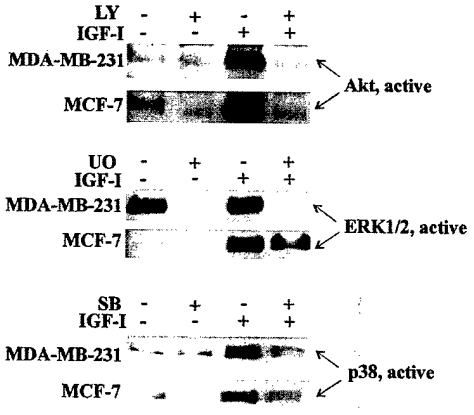

Fig. 7. PI-3K and MAPK inhibitors. Synchronized cultures of MDA-MB-231 and MCF-7 cells were treated with LY294002, UO126, or SB203580 in the presence or absence of IGF-I, as described in "Materials and Methods." The activities of Akt, ERK1/ERK2, and p38 kinases were determined by WB in 50 μg of protein lysates using specific antibodies. Representative results are shown.

migration of ER-positive and ER-negative cells; in fact, the compound stimulated cell motility. The addition of IGF-I as a chemoattractant significantly counteracted the effects of all three inhibitors; however, no clear association between the cellular levels of the IGF-IR and this competing action of IGF-I was noted (Table 4). These results suggested that IGF-I-dependent motility in both types of cells requires the PI-3K and p38 pathways but does not rely on the activity of ERK1/ERK2.

DISCUSSION

The experimental and clinical evidence supports the notion that hyperactivation of the IGF-IR may be critical in early steps of breast cancer development, promoting cell growth, survival, and resistance to therapeutic treatments. However, the function of the IGF-IR in the later stages of the disease, including metastasis, is still obscure (1). For instance, whereas the IGF-IR has been found overexpressed in primary breast tumors, its levels, similar to ER levels, appear to undergo reduction during the course of the disease (1, 18). According to Pezzino *et al.* (41), who studied the IGF-IR status in two patient subgroups representing either a low risk (ER- and progesterone receptor-positive, low mitotic index, diploid) or a high risk (ER- and progesterone receptor-negative, high mitotic index, aneuploid) population, there is a highly significant correlation between IGF-IR expression and better prognosis. Similar conclusions were reached by Peyrat and Bonneterre (42) and recently by Schnarr *et al.* (18). Therefore, it has been proposed that similar to the ER, the IGF-IR marks more differentiated tumors with better clinical outcome. However, it has also been argued that the IGF-IR may play a role in early steps of tumor spread because node-positive/IGF-IR-positive tumors appeared to have a worse prognosis than node-negative/IGF-IR-positive tumors (1, 42). In addition, quite rare cases of ER-negative but IGF-IR-positive tumors are associated with shorter disease-free survival (43).

In breast cancer cell lines, a hormone-dependent and less aggressive phenotype correlates with a good IGF-IR expression (1, 19, 42). By contrast, different ER-negative, breast cancer cell lines express low levels of the IGF-IR and generally do not respond to IGF-I with growth (1, 18–22). However, many ER-negative cell lines appear to depend on the IGF-IR for tumorigenesis and metastasis. For instance, blockade of the IGF-IR in MDA-MB-231 cells by anti-IGF-IR antibody reduced migration *in vitro* and tumorigenesis *in vivo*, and expression of a soluble IGF-IR in MDA-MB-435 cells impaired growth, tumorigenesis, and metastasis in animal models (1, 14, 16, 23). These observations suggest that some growth-unrelated pathways of the IGF-IR may be operative in the context of ER-negative cells.

Here we studied whether this particular IGF-I dependence of ER-negative breast cancer cells relates to the nonmitogenic function of the IGF-IR, such as cell migration. Our experiments indicated that the IGF-IR is an effective mediator of cell motility. Furthermore, IGF-I-induced migration was proportional to IGF-IR content. We demonstrated, for the first time, that in MDA-MB-231 ER-negative cells, IGF-IR signaling pathways responsible for cell movement include PI-3K and p38 kinases. Indeed, an acute IGF-I stimulation of MDA-MB-231 and MDA-MB-231/IGF-IR cells appears to induce both PI-3K and p38 kinases, suggesting that this short-time activation may be involved in migration. Both of these pathways have been shown previously to regulate cell motility in breast cancer cells and other cell types (35, 44). Interestingly, the migration of both ER-negative and ER-positive cells was enhanced by a specific MEK1/MEK2 inhibitor UO126. We observed this effect over a broad range of UO126 doses (1–10 μM) and in several MDA-MB-231- and MCF-7-derived clones; the same doses always suppressed cell proliferation in serum-containing medium and PRF-SFM (data not shown and Table 2). These

Table 3 *IGF-I-induced migration in ER-negative and ER-positive breast cancer cells*

The IGF-I-induced migration of MDA-MB-231 and MCF-7 cells, their IGF-IR-overexpressing derivatives, as well as control clones MDA-MB-231/5M and MCF-7/pc2, was determined after 24 hr, as described in "Materials and Methods." At this time point, IGF-I did not produce statistically significant differences in the growth and survival of the cells studied (Fig. 3). Migration (%) represents the difference (in %) between basal migration in PRF-SFM and migration in the presence of IGF-I. The chemotaxis results are averages (±SE) from at least nine experiments. The chemokinesis results are averages (±SE) from three experiments.

Cell line	Migration (%)[a]		
	Lower well	Upper well	Both wells
MDA-MB-231	+24 ± 3.9	+2 ± 0.1	+7 ± 0.4
MDA-MB-231/IGF-IR	+79 ± 4.1	+10 ± 0.1	+12 ± 0.5
MDA-MB-231/5M	+20 ± 2.0	+1 ± 0.0	+5 ± 0.1
MCF-7	+23 ± 4.7	−1 ± 0.0	−2 ± 0.0
MCF-7/IGF-IR	+47 ± 5.6	−10 ± 0.5	−8 ± 0.2
MCF-7/pc2	+17 ± 3.1	0 ± 0.0	−1 ± 0.0

[a] IGF-I.

Table 2 *Effects of PI-3K and MAPK inhibitors on growth and survival of ER-negative and ER-positive breast cancer cells*

MDA-MB-231 cells, MDA-MB-231/IGF-IR clone 31, MCF-7 cells, and MCF-7/IGF-IR clone 12 were cultured in PRF-SFM with or without IGF-I in the presence or absence of the inhibitors for 48 h, as described in "Materials and Methods." The difference (in percentages) between the number of live cells under treatment and the number of cells cultured under control conditions (without inhibitors) was defined as inhibition (%). The results are averages from three experiments; SDs are given.

Cell line	Inhibition (%)					
	LY294002 (PI-3K)		UO126 (MEK)		SB203580 (p38)	
	SFM	+IGF	SFM	+IGF	SFM	+IGF
MDA-MB-231	9.4 ± 1.0	7.8 ± 0.8	35.0 ± 2.6	39.0 ± 2.7	47.8 ± 2.2	42.5 ± 4.4
MDA-MB-231/IGF-IR	11.1 ± 1.2	12.3 ± 0.9	18.3 ± 0.9	22.9 ± 1.3	29.5 ± 2.0	35.6 ± 3.6
MCF-7	68.8 ± 3.3	35.0 ± 1.2	42.6 ± 3.8	26.3 ± 2.5	11.7 ± 1.2	10.0 ± 0.4
MCF-7/IGF-IR	73.2 ± 6.7	34.6 ± 2.7	49.4 ± 3.9	20.2 ± 1.5	24.7 ± 0.2	25.9 ± 0.9

Table 4 *Effects of PI-3K and MAPK inhibitors on migration of ER-negative and ER-positive breast cancer cells*

The migration of MDA-MB-231 cells, MDA-MB-231/IGF-IR clone 31, MCF-7 cells, and MCF-7/IGF-IR clone 12 was tested in modified Boyden chambers as described in "Materials and Methods." The inhibitors were added to the upper wells at the time of cell plating, and their effect on basal (SFM) or IGF-I-induced (+IGF) migration was assessed after 24 h (for UO126 and SB203580) or 12 h (for LY94002). At these time points, the compounds did not affect cell growth and survival. The values represent the percentage of change relative to the migration at basal conditions in PRF-SFM (SFM) without inhibitors or chemoattractants. The experiments were repeated at least three times; the average results (±SD) are given.

	Change (%)					
	LY294002 (PI-3K)		UO126 (MEK)		SB203580 (p38)	
Cell line	SFM	+IGF	SFM	+IGF	SFM	+IGF
MDA-MB-231	−45.1 ± 1.1	−18.9 ± 0.9	+53.4 ± 3.5	+36.4 ± 2.2	−30.2 ± 2.9	−8.5 ± 0.7
MDA-MB-231/IGF-IR	−38.3 ± 3.5	−12.2 ± 0.4	+29.0 ± 2.0	+12.6 ± 0.7	−40.1 ± 0.4	−2.5 ± 0.0
MCF-7	−24.7 ± 1.2	−9.6 ± 0.9	+94.9 ± 3.9	+56.4 ± 1.7	−18.9 ± 1.1	−5.6 ± 0.2
MCF-7/IGF-IR	−20.4 ± 1.0	−8.0 ± 0.7	+65.6 ± 5.4	+23.8 ± 1.9	−24.8 ± 0.8	−1.7 ± 0.1

peculiar effects suggest that MEK1/2 may represent a regulatory point balancing mitogenic and nonmitogenic cell responses.

In contrast with the positive effects of IGF-I on cell motility in ER-negative and ER-positive breast cancer cells, this growth factor never stimulated the proliferation of MDA-MB-231 cells, whereas it induced the growth of MCF-7 cells and MCF-7-derived clones overexpressing the IGF-IR. It is has been established by Rubini *et al.* (30) and Reiss *et al.* (31) that mitogenic response to IGF-I requires a threshold level of IGF-IR expression (in fibroblasts, $\sim 1.5 \times 10^4$). Here, we demonstrated that increasing the levels of the IGF-IR from $\sim 7 \times 10^3$ up to $\sim 2.5 \times 10^5$ and subsequent up-regulation of IGF-IR tyrosine phosphorylation was not sufficient to induce IGF-I-dependent growth of MDA-MB-213 cells. Similar results were obtained by Jackson and Yee (21), who showed that overexpression of IRS-1 in ER-negative MDA-MB-435A and MDA-MB-468 breast cancer cells did not stimulate IGF-I-dependent mitogenicity. These authors suggested that the lack of IGF-I response, even in IRS-1-overexpressing ER-negative cells, was related to insufficient stimulation of ERK1/ERK2 and PI-3K pathways (21). Defective insulin response in ER-negative cell lines has also been described by Costantino *et al.* (45) and linked with an increased tyrosine phosphatase activity.

Our experiments suggested that the lack of IGF-I mitogenicity in MDA-MB-231 and MDA-MB-231/IGF-IR cells was not related to the impaired IGF-IR or IRS-1 tyrosine phosphorylation. The cells were also able to respond to an acute IGF-I stimulation with a marked activation of the PI-3K/Akt and ERK-1/ERK2 pathways. We hypothesize that this transient stimulation could be sufficient to induce some IGF-I response, such as cell motility. Mitogenic response, on the other hand, may rely on a more sustained activation of critical IGF-IR signals, as demonstrated before with mouse embryo fibroblasts (36). Indeed, the most significant difference in IGF-I signal between ER-negative and ER-positive cells rested in the impaired long-term stimulation of the PI-3K/Akt pathway; MDA-MB-231 and MDA-MB-231/IGF-IR cells were unable to sustain this IGF-I-induced signal for 1 or 2 days, whereas in MCF-7 and MCF-7/IGF-IR cells, the PI-3K/Akt pathway was still active at this time. The subsequent experiments with MDA-MB-231 cells transfected with constitutively active Akt mutants demonstrated that the increased biological activity of Akt was not sufficient to completely reverse cell death in PRF-SFM (Fig. 6*C* and data not shown). This suggested that although a sustained Akt activity could be important in the survival of breast cancer cells, other pathways, or a proper equilibrium between Akt and other pathways (such as ERK1/2), are also critical. The latter possibility could be supported by our finding that hyperactivation of Akt down-regulates the ERK1/2 pathway. Normally, this pathway appears to play a role in the survival of ER-negative cells (Table 2).

In summary, our data suggest that IGF-IR signaling and function may be different in hormone-dependent and -independent breast cancer cells. In ER-positive MCF-7 cells, IGF-IR transmits various signals, such as growth, survival, migration, and adhesion. In ER-negative MDA-MB-231 cells, the growth-related functions of the IGF-IR become attenuated, but the receptor is still able to control nonmitogenic processes, such as migration. It is likely that this kind of evolution is also involved with the response to other growth factors. Epidermal growth factor, for instance, is an effective mitogen for ER-positive breast cancer cells but does not stimulate the proliferation or survival in MDA-MB-231 cells, despite high EGF-R expression (46). However, as demonstrated recently by Price *et al.* (46), EGF is a potent chemoattractant for MDA-MB-231 cells. EGF-induced migration in MDA-MB-231 cells requires PI-3K and phospholipase Cγ and is not inhibited by antagonists of ERK1/ERK2.

In conclusion, mitogenic and nonmitogenic pathways induced by growth factors in breast cancer cells may be dissociated, and attenuation of one is not necessarily linked with the cessation of the other. Delineating the nonmitogenic responses will be critical for the development of drugs specifically targeting metastatic cells.

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IGF-I Receptor-induced Cell-Cell Adhesion of MCF-7 Breast Cancer Cells Requires the Expression of Junction Protein ZO-1*

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Hyperactivation of the insulin-like growth factor I receptor (IGF-IR) contributes to primary breast cancer development, but the role of the IGF-IR in tumor metastasis is unclear. Here we studied the effects of the IGF-IR on intercellular connections mediated by the major epithelial adhesion protein, E-cadherin (E-cad). We found that IGF-IR overexpression markedly stimulated aggregation in E-cad-positive MCF-7 breast cancer cells, but not in E-cad-negative MDA-MB-231 cells. However, when the IGF-IR and E-cad were co-expressed in MDA-MB-231 cells, cell-cell adhesion was substantially increased. The IGF-IR-dependent cell-cell adhesion of MCF-7 cells was not related to altered expression of E-cad or α-, β-, or γ-catenins but coincided with the up-regulation of another element of the E-cad complex, zonula occludens-1 (ZO-1). ZO-1 expression (mRNA and protein) was induced by IGF-I and was blocked in MCF-7 cells with a tyrosine kinase-defective IGF-IR mutant. By co-immunoprecipitation, we found that ZO-1 associates with the E-cad complex and the IGF-IR. High levels of ZO-1 coincided with an increased IGF-IR/α-catenin/ZO-1-binding and improved ZO-1/actin association, whereas down-regulation of ZO-1 by the expression of an anti-ZO-1 RNA inhibited IGF-IR-dependent cell-cell adhesion. The results suggested that one of the mechanisms by which the activated IGF-IR regulates E-cad-mediated cell-cell adhesion is overexpression of ZO-1 and the resulting stronger connections between the E-cad complex and the actin cytoskeleton. We hypothesize that in E-cad-positive cells, the IGF-IR may produce antimetastatic effects.

The insulin-like growth factor I (IGF-I)[1] receptor (IGF-IR) is a ubiquitous tyrosine kinase capable of regulating different growth-related and -unrelated processes (1–3). Recent evidence indicates that the IGF-IR may be involved in breast cancer development. The IGF-IR is significantly (10–14-fold) overexpressed in estrogen receptor-positive primary breast tumors compared with normal mammary epithelium or benign tumors (1, 4). Moreover, the intrinsic ligand-independent tyrosine kinase activity of the IGF-IR has been found to be substantially up-regulated (~2–4-fold) in breast cancer cells (4). It has been suggested that the increased receptor function coupled with enhanced receptor expression amounts to a 40-fold elevation in IGF-IR activity in estrogen receptor-positive breast tumors (4). Recent clinical and experimental data indicate that up-regulation of IGF-IR signaling in estrogen receptor-positive breast cancer cells is associated with autonomous cell proliferation, estrogen-independence, and increased resistance to various antitumor treatments (1). Consequently, it is believed that hyperactivation of the IGF-IR may induce and sustain the growth of primary breast tumors (1).

The role of the IGF-IR in breast cancer metastasis, however, is unclear. The experimental data suggest that the IGF-IR has a function in cell spreading by effectively stimulating the motility of different metastatic breast cancer cell lines lacking the expression of a major adhesion protein, E-cadherin (E-cad) (1, 5, 6). On the other hand, we and others have shown that in more differentiated E-cad-positive cells, IGF-I treatment or IGF-IR overexpression up-regulates cell-cell adhesion, which correlates with increased cell survival in three-dimensional culture and with reduced cell migration *in vitro* and in organ culture (1, 7–10).

The mechanism of IGF-I-dependent intercellular adhesion and the clinical consequences of this phenomenon have not been fully elucidated. Previously, we demonstrated that in MCF-7 human breast cancer cells, the IGF-IR co-localizes and co-precipitates with the E-cad complex and that IGF-induced aggregation is blocked with an anti-E-cad antibody (7). In this study we assessed the effects of the IGF-IR on the elements of the E-cad adhesion complex, *i.e.* E-cad; β-, γ-, and α-catenins; and α-catenin-associated proteins (see Fig. 6). The initial results prompted us to focus on an α-catenin-binding element, the junction protein zonula occludens-1 (ZO-1).

ZO-1 is a ~220-kDa scaffolding protein containing various domains (an SH3 domain, three PDZ domains, a proline-rich region, and a guanylate kinase domain) that allow its interaction with specialized sites of plasma membrane as well as with other proteins (11, 12). ZO-1 is a characteristic element of tight junctions, but recently its presence has also been demonstrated in E-cad adherens junctions (13–15). The role of ZO-1 in adherens junctions is yet unclear, but it is assumed that it may functionally link E-cad with the actin cytoskeleton because it associates with α-catenin and actin through its N and C terminus, respectively (Ref. 13, see Fig. 6). In addition, as a member of the membrane-associated guanylate kinase homologue (MAGUK) family of putative signaling proteins, ZO-1 may be involved in signal transduction. Indeed, ZO-1 has been found to

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[1] The abbreviations used are: IGF-I, insulin-like growth factor I; IGF-IR, IGF-I receptor; E-cad, E-cadherin; ZO-1, zonula occludens-1 junction protein; Y3F, Tyr-1131, Tyr-1135, and Tyr-1136 replaced with Phe; WB, Western blotting or Western blotted; pAb, polyclonal antibody; mAb, monoclonal antibody; ERK1, extracellular signal-related kinase 1; IRS-1, insulin receptor substrate 1; MAPK, mitogen-activated protein kinase.

TABLE I

Effects of IGF-IR and ZO-1 expression on cell aggregation in E-cad-positive and -negative breast cancer cells

The stable cell lines (MCF-7, MCF-7/IGF-IR, MCF-7/IGF-IR/anti-ZO-1, MCF-7/Y3F, MDA-MB-231, and MDA-MB-231/IGF-IR) and transiently transfected populations (MDA-MB-231/E-cad, MDA-MB-231/vector, MDA-MB-231/IGF-IR/E-cad, and MDA-MB-231/IGF-IR/vector) were cultured as three-dimensional spheroids in normal growth medium. The number of spheroids of different sizes was established as described under "Materials and Methods." The values represent a sum of spheroids in 10 optical fields under × 10 magnification. The results are mean ± S.E. from at least three experiments. Representative three-dimensional cultures are shown in Figs. 1 and 7.

Cells	Spheroids		
	25 ≤ 50 μm	50 ≤ 100 μm	>100 μm
MCF-7	21.0 ± 1.9	96.0 ± 6.7	2.7 ± 0.9
MCF-7/IGF-IR	1.7 ± 0.7	22.3 ± 1.4	86.5 ± 3.9
MCF-7/IGF-IR/anti-ZO-1	75.0 ± 3.5	40.7 ± 2.1	0.0 ± 0.0
MCF-7/Y3F	45.0 ± 2.9	39.8 ± 4.6	0.0 ± 0.0
MDA-MB-231	7.0 ± 0.6	0.5 ± 0.2	0.0 ± 0.0
MDA-MB-231/IGF-IR	12.5 ± 0.8	0.5 ± 0.1	0.0 ± 0.0
MDA-MB-231/E-cad	39.8 ± 3.6	15.2 ± 1.1	0.0 ± 0.0
MDA-MB-231/vector	10.0 ± 1.4	0.6 ± 0.4	0.0 ± 0.0
MDA-MB-231/IGF-IR/E-cad	27.0 ± 2.2	68.3 ± 7.2	8.0 ± 1.5
MDA-MB-231/IGF-IR/vector	18.6 ± 2.2	0.9 ± 0.3	0.0 ± 0.0

bind a target of Ras, AF6 (16). Deletions or mutations in the *ZO-1* gene produced overgrowth, suggesting that ZO-1 may act as a tumor suppressor (11). In breast cancer, ZO-1 is usually co-expressed with E-cad and is a strong independent marker of a more differentiated phenotype (17).

At present, very little is known about the regulation of ZO-1 by growth factors. However, some recent studies demonstrated that epidermal growth factor and vascular endothelial growth factor are able to increase ZO-1 tyrosine phosphorylation, modulate its subcellular localization, and consequently produce increased permeability (18–20). Here, we present the first evidence that in MCF-7 breast cancer cells 1) the IGF-IR up-regulates ZO-1 expression, 2) elevated levels of ZO-1 coincide with enhanced IGF-IR/E-cad-mediated cell-cell adhesion, and 3) ZO-1 expression is required for IGF-IR-increased cell aggregation in E-cad-positive MCF-7 cells.

MATERIALS AND METHODS

Expression Plasmids

E-cad Expression Plasmid—The pBAT-EM2 plasmid is a derivative of pBR322 and contains the mouse E-cad cDNA cloned under the β-actin promoter in pBR322 (21). As demonstrated previously, transfection of MDA-MB-231 cells with pBAT-EM2 results in E-cad overexpression, improved cell aggregation, and reduced metastatic potential of the cells (21, 22).

Antisense ZO-1 RNA Vector—The pcDNA3/anti-ZO-1 plasmid encoding the anti-ZO-1 antisense RNA contains a 959-base pair *Bam*HI fragment of the human ZO-1 cDNA (nucleotides 4205–5164) inserted (in the 3′-5′ orientation) into the pcDNA3.1/hygro plasmid (Invitrogen). pcDNA3/sense-ZO-1 is the control vector in which the above 959-base pair ZO-1 cDNA fragment was cloned in the 5′-3′ orientation.

Cell Lines and Cell Culture Conditions

MCF-7/IGF-IR clones 12, 15, and 17 are MCF-7-derived clones overexpressing the IGF-IR at the levels 5×10^5, 3×10^6, and 1×10^6 receptors/cell, respectively (7). To avoid clonal variation, in several experiments we used a population of mixed clones 12, 15, and 17. The mixed population is referred to as MCF-7/IGF-IR cells and expresses ~0.9×10^6 IGF-IR receptors/cell (which represents ~18-fold overexpression over the levels in normal cells) (1). MCF-7/IGF-IR/Y3F express an IGF-IR (~3×10^6 receptors/cell) with inactivating mutations in the tyrosine kinase domain (Tyr-1131, Tyr-1135, and Tyr-1136 replaced with Phe) (23). MCF-7/IGF-IR/Y3F cells were derived from MCF-7 cells by stable transfection with the pcDNA3/IGF-IR/KR plasmid and subsequent selection in 2 mg/ml G418. The results obtained with the MCF-7/IGF-IR/Y3F clone were verified using a population of MCF-7 cells transiently transfected with the IGF-IR/Y3F vector (see below). MCF-7/IGF-IR/anti-ZO-1 and MCF-7/IGF-IR/sense ZO-1 cells were derived from MCF-7/IGF-IR clone 15 by stable transfection with the antisense and sense ZO-1 vectors, respectively and subsequent selection in 500 μg/ml hygromycin B.

MDA-MB-231 is a metastatic breast cancer cell line lacking E-cad and expressing ~7×10^3 IGF-IR receptors/cell (24).[2] MDA-MB-231/IGF-IR clone 31 was derived from MDA-MB-231 cells by stable transfection with the pcDNA3/IGF-IR plasmid. MDA-MB-231/IGF-IR cells express ~250,000 IGF-IR/cell.[2]

All cell lines were grown in Dulbecco's modified Eagle's medium/F12 (1:1) containing 5% calf serum. MCF-7- and MDA-MB-231-derived clones transfected with the wild-type or mutant IGF-IR were maintained in growth medium with 100 μg/ml G418. MCF-7/IGF-IR/anti-ZO-1 and MCF-7/IGF-IR/sense ZO-1 cells were cultured in growth medium with 50 μg/ml hygromycin B. In the experiments requiring serum-free conditions, the cells were cultured in phenol red-free Dulbecco's modified Eagle's medium containing 0.5 mg/ml bovine serum albumin, 1 μM $FeSO_4$, and 2 mM L-glutamine (referred to as SFM).

Transient Transfection

MDA-MB-231 and MDA-MB-231/IGF-IR cells were transiently transfected using LipofectAMINE 2000 (Life Technologies, Inc.) (reagent/DNA ratio, 5 μl/1 μg). The transfection was carried out in growth medium for 24 h, and then the cells were lysed and processed for E-cad Western blotting (WB). To evaluate the extent of cell-cell adhesion in the transfected MDA-MB-231 and MDA-MB-231/IGF-IR cells, the cells were trypsinized upon transfection, counted, and placed in three-dimensional suspension culture as described below. MCF-7 cells were transfected for 6 h in growth medium using Fugene 6 (Roche Molecular Biochemicals) (reagent/DNA ratio, 3 μl/1 μg). To study IGF-I signaling, the transfected MCF-7 cells were shifted to SFM for 36 h and stimulated with IGF-I for 15 min. The efficiency of transfection (transfected cells/total cell number) was at least 70% for all cell types and was estimated by scoring fluorescent cells in cultures transfected with the plasmid pCMS (encoding green fluorescent protein) (Invitrogen).

Three-dimensional Spheroid Culture

The cells were grown to 70–80% confluence, trypsinized, and plated in single-cell suspension in 2%-agar-coated plates containing either normal growth medium or SFM. 2×10^6 cells were plated per 100 mm culture dish. To generate three-dimensional spheroids, the plates were rotated for 4 h at 37 °C. The spheroids started to assemble at ~1 h after plating and were completely organized after 3–4 h of culture in suspension. The three-dimensional cultures were photographed using a phase-contrast microscope (Nikon or Olympus). The extent of aggregation was scored by measuring the spheroids with an ocular micrometer. For each cell type, the spheroids between 25 and 50, 50 and 100, and >100 μm (in the smallest cross-section) were counted in 10 different fields under × 10 magnification.

IGF Stimulation

70% confluent cell cultures were synchronized in SFM for 36 h and then stimulated with 20 ng/ml IGF-I for 0–72 h.

Immunoprecipitation and Western Blotting

The expression of different elements of the adhesion complex was assessed in 500 μg of protein lysate by immunoprecipitation and WB

[2] Bartucci, M., Morelli, C., Mauro, L., Ando', S., and Surmacz, E. (2001) *Cancer Res.* **61,** 6747–6754.

with appropriate antibodies. The expression of ERK1/ERK2 was tested in 50 μg of total cell lysate. The cell lysis buffer contained 50 mM HEPES, pH 7.5, 150 mM NaCl, 1% Triton X-100, 1.5 mM $MgCl_2$, 1 mM $CaCl_2$, 100 mM NaF, 0.2 mM Na_3VO_4, 1% phenylmethylsulfonyl fluoride, and 1% aprotinin as described before (25). The following antibodies were used: anti-ZO-1 polyclonal antibody (pAb) (Zymed Laboratories Inc.) for ZO-1 immunoprecipitation (5 μg/ml) and WB (2 μg/ml); anti-E-cad monoclonal antibody (mAB), clone 36 (Transduction Laboratories) for E-cadherin immunoprecipitation (2 μg/ml) and WB (0.1 μg/ml); anti-α-catenin pAb (Sigma or Zymed Laboratories Inc.) for α-catenin immunoprecipitation (4 μg/ml) and WB (anti-serum dilution 1:4000); anti-β-catenin mAb (Transduction Laboratories) for β-catenin immunoprecipitation (4 μg/ml) and WB (0.5 μg/ml); anti-γ catenin pAb (Sigma) for γ-catenin WB (1 μg/ml); anti-actin mAb clone AC-40 (Sigma) for actin WB (0.4 μg/ml); anti-IGF-IR mAb, clone αIR-3 (Calbiochem) for IGF-IR immunoprecipitation (3 μg/ml), and anti-IGF-IR pAb C-20 (Santa Cruz Biotechnology) for IGF-IR WB (0.2 μg/ml); anti-p85 pAb (Upstate Biotechnology, Inc.) for the p85 subunit of the phosphatidylinositol 3-kinase WB (0.25 μg/ml); anti-phospho-MAPK mAb (New England Biolabs, Inc.) for active ERK1/ERK2 WB (0.5 μg/ml); and anti-MAPK pAb (New England Biolabs) for total ERK1/ERK2 WB (1 μg/ml). Tyrosine phosphorylation of immunoprecipitated proteins was measured by WB with anti-phosphotyrosine mAb (Transduction Laboratories) (0.03 μg/ml). Western blots were developed using an ECL chemiluminescence kit (Amersham Pharmacia Biotech). The intensity of bands representing relevant proteins was measured by laser densitometry scanning.

RESULTS

IGF-IR Overexpression Stimulates Cell-Cell Adhesion through an E-cad-dependent Mechanism—First, we demonstrated that under three-dimensional culture conditions, overexpression of the IGF-IR stimulated cell-cell adhesion in E-cad-positive MCF-7 breast cancer cells but not in E-cad-negative MDA-MB-231 cells (Fig. 1, *A* and *B*) (Table I). However, coexpression of the IGF-IR and E-cad resulted in robust cell-cell adhesion of MDA-MB-231 cells, whereas the expression of E-cad alone was less efficient in inducing intercellular contacts (Fig. 1, *C* and *D*) (Table I). These results, together with our previous data showing that IGF-IR-mediated aggregation in MCF-7 cells is blocked with an anti-E-cad antibody (7), indicated that IGF-IR adhesion signals are transmitted through the E-cad complex.

IGF-IR Overexpression Up-regulates ZO-1—We tested whether high levels of the IGF-IR affect the expression of the proteins within the E-cad complex and found that in MCF-7 and MCF-7/IGF-IR cells cultured as three-dimensional spheroids, the levels of E-cad and α-, β-, and γ-catenin were similar. However, the abundance of ZO-1 was significantly increased in MCF-7/IGF-IR cells (Fig. 2). The tyrosine phosphorylation of all these adhesion proteins was undetectable in spheroids and was not influenced by IGF-IR overexpression (Fig. 5 and data not shown).

To investigate whether the increased expression of ZO-1 in MCF-7/IGF-IR cells depends on IGF-IR tyrosine kinase activity, we generated by stable or transient transfection MCF-7/IGF-IR/Y3F cells expressing high levels of a kinase-defective IGF-IR mutant (IGF-IR/Y3F). The overexpression of the IGF-IR/Y3F mutant resulted in impaired IGF-I response, which was reflected by markedly reduced IGF-IR and IRS-1 tyrosine phosphorylation, decreased IRS-1/p85 binding, and diminished ERK1/ERK2 stimulation (Fig. 3). The basal expression of ZO-1 in MCF-7/IGF-IR/Y3F cells was significantly reduced compared with that in MCF-7/IGF-IR cells, indicating that tyrosine kinase activity of the IGF-IR is required for the up-regulation of ZO-1 (Fig. 3). Interestingly, the inhibition of IGF-I response did not affect E-cad expression, suggesting a selective action of the IGF-IR toward ZO-1 (Fig. 3). The blockade of the IGF-IR signal in MCF-7/IGF-IR/Y3F cells coincided with reduced cell-cell adhesion (Table 1).

ZO-1 mRNA and Protein Expression Is Regulated by IGF-I—To establish whether the activation of the IGF-IR by IGF

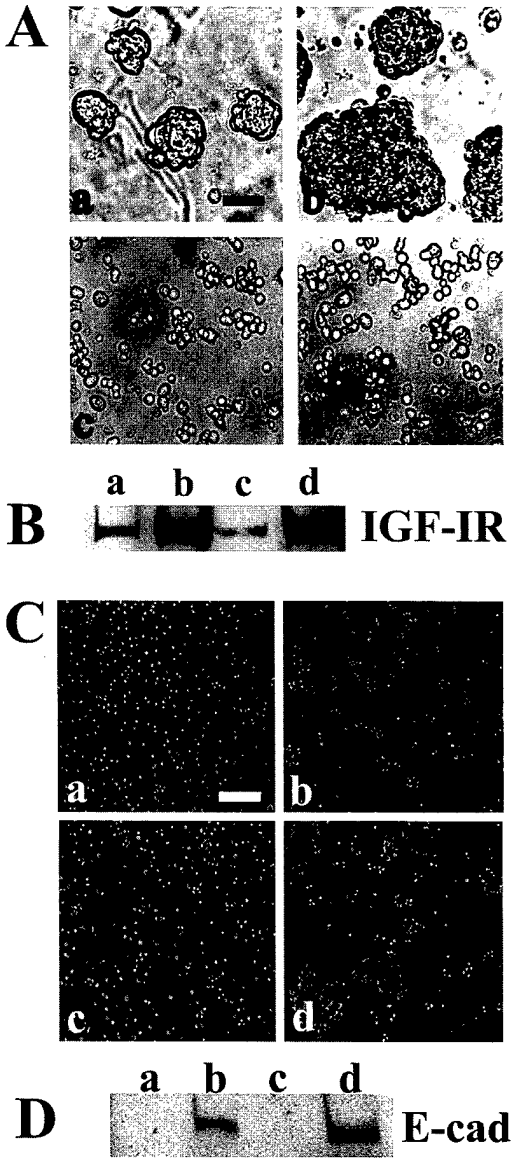


FIG. 1. **IGF-IR overexpression stimulates cell-cell adhesion in E-cad-positive but not in E-cad-negative breast cancer cells.** *A*, E-cad-positive MCF-7 and MCF-7/IGF-IR cells and E-cad-negative MDA-MB-231 and MDA-MB-231/IGF-IR cells were cultured in normal growth medium as three-dimensional spheroids for 24 h as described under "Materials and Methods" and then photographed under phase contrast microscopy. *a*, MCF-7 cells expressing ~6×10^4 IGF-IR/cell (7); *b*, MCF-7/IGF-IR, clone 12 expressing ~5×10^5 IGF-IGF-IR/cell (7); *c*, MDA-MB-231 cells with ~7×10^3 IGF-IGF-IR/cell (24), and *d*, MDA-MB-231/IGF-IR, clone 31 with ~3×10^5 IGF-IR/cell.[2] The *bar* in *a* equals 50 μm. *B*, IGF-IR levels in cells pictured in Fig. 1*A*, *a–d* were assessed by WB in 50 μg of cell lysate as described under "Materials and Methods." *C*, MDA-MB-231 and MDA-MB-231/IGF-IR cells were transiently transfected with the E-cad expression plasmid (*b* and *d*) or a vector alone (*a* and *c*) and then cultured in suspension as three-dimensional spheroids. The *bar* in *a* equals 100 μm. *D*, E-cad levels in cells pictured in Fig. 1*C*, *a–d* were determined by WB in 50 μg of cell lysate, as described under "Materials and Methods."

produces a similar effect on ZO-1 as that seen with IGF-IR overexpression, we studied ZO-1 mRNA and protein in MCF-7 and MCF-7/IGF-IR cells treated with 20 ng/ml IGF-I for 1–72 h (Fig. 4). In MCF-7 cells cultured in SFM, the basal levels of ZO-1 mRNA were low and were markedly increased between 4 and 36 h of IGF-I treatment (Fig. 4*A*). In contrast, the abundance of ZO-1 mRNA was always elevated in MCF-7/IGF-IR cells and was only moderately improved by IGF-I (4–72 h) (Fig.

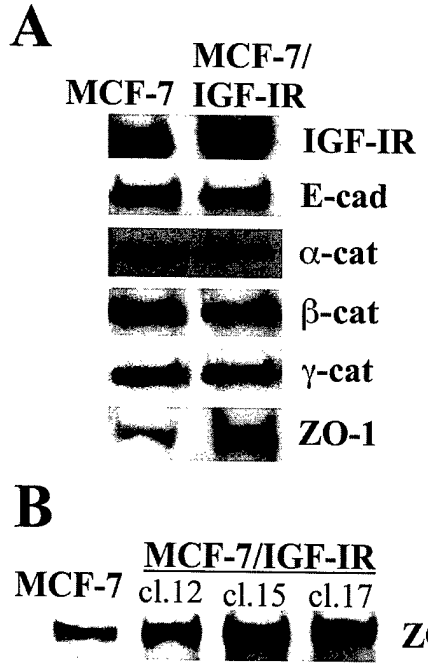


FIG. 2. **Expression of adhesion proteins in MCF-7 and MCF-7/IGF-IR cells.** The expression of adhesion proteins and the IGF-IR was studied in 50 μg of protein lysates obtained from cells cultured as three-dimensional spheroids in normal growth medium. MCF-7/IGF-IR cells are pooled MCF-7/IGF-IR clones 12, 15, and 17 (see "Materials and Methods"). *A*, the levels of the IGF-IR; E-cad; α-, β-, γ-catenin (*cat*); and ZO-1 detected by WB using specific antibodies (see "Materials and Methods"). *B*, the expression of ZO-1 in MCF-7 cells and in MCF-7/IGF-IR clones (*cl.*) 12, 17, and 15, expressing ~5 × 10^5, 1 × 10^6, and 3 × 10^6 IGF-IR/cell, respectively (7).

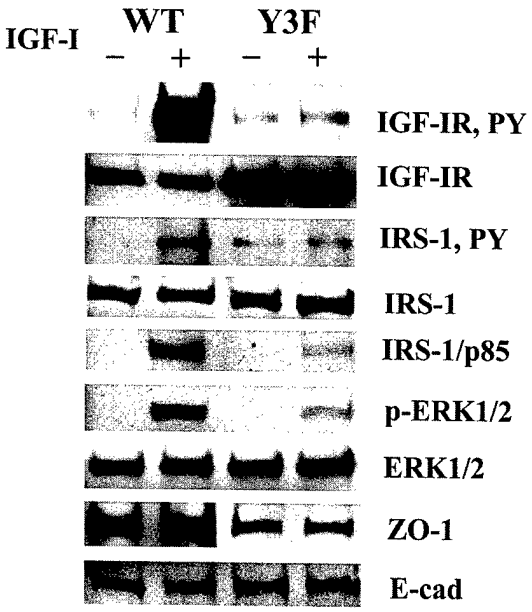


FIG. 3. **ZO-1 expression is inhibited in MCF-7/IGF-IR/Y3F cells.** MCF-7/IGF-IR cells expressing the wild-type IGF-IR (*WT*) and MCF-7/IGF-IR/Y3F cells stably transfected with a dominant-negative kinase-defective IGF-IR mutant (*Y3F*) were synchronized in SFM for 36 h and then stimulated for 15 min with 20 ng/ml IGF-I as described under "Materials and Methods." The expression and tyrosine phosphorylation (*PY*) of the IGF-IR and IRS-1 in the cells were detected by immunoprecipitation and WB in 500 μg of protein lysates. The binding of the p85 subunit of the phosphatidylinositol 3-kinase to IRS-1 (IRS-1/p85) was studied by WB in IRS-1 immunoprecipitates. The expression of active ERK1/ERK2 (p-ERK1/2), total ERK1/2, ZO-1, and E-cad was evaluated by WB in 50 μg of total protein lysates. The specific antibodies used are listed under "Materials and Methods." Similar results were obtained with MCF-7 cells transiently transfected with the IGF-IR/Y3F expression vector.

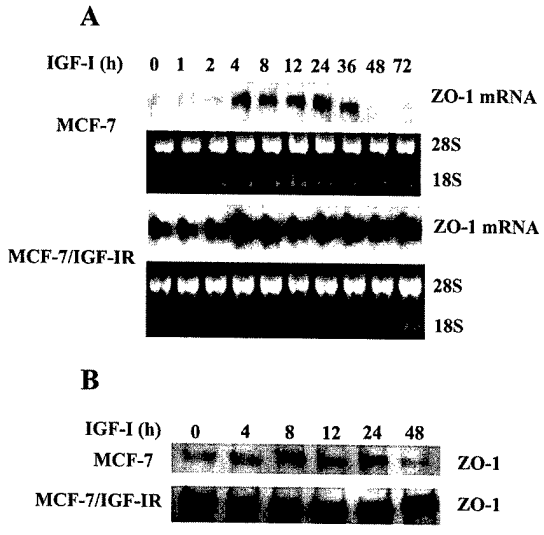
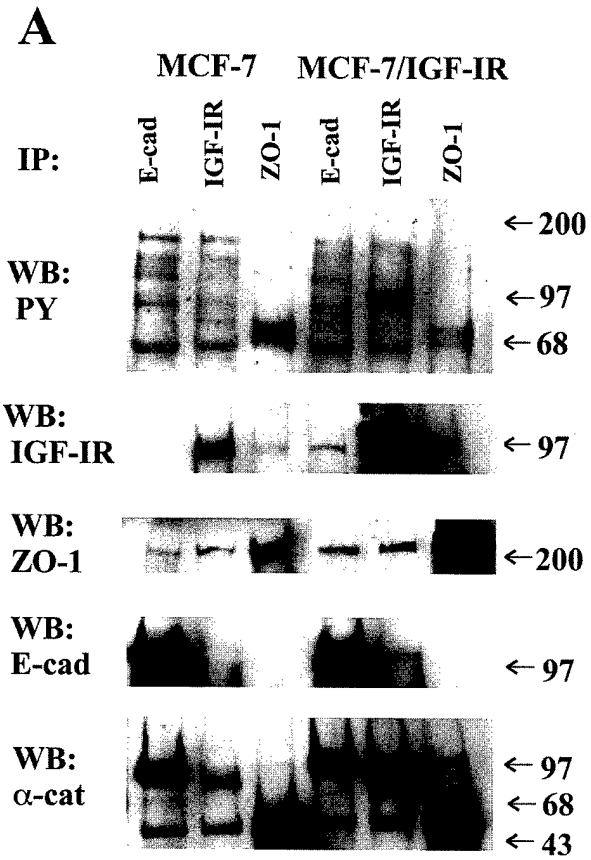
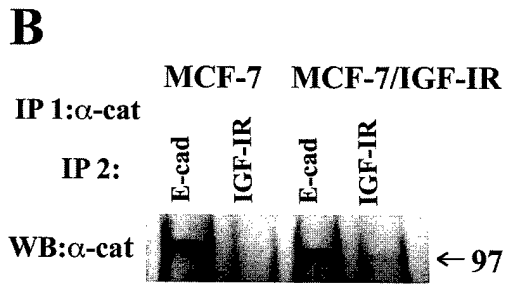


FIG. 4. **ZO-1 mRNA and protein are regulated by IGF-I.** *A*, MCF-7 cells and MCF-7/IGF-IR were synchronized in SFM (time 0) and then stimulated with 20 ng/ml IGF-I for different times (1–72 h). The expression of 7.8-kilobase ZO-1 mRNA in MCF-7 and MCF-7/IGF-IR cells was studied by Northern blotting in 20 μg of total RNA using a [^{32}P]dCTP-labeled ZO-1 probe (described under "Materials and Methods"). 28 and 18 S rRNA are shown as a control of RNA loading. *B*, the expression of ZO-1 protein in IGF-I-treated cells was detected by WB as described under Fig. 3.

4*A*). ZO-1 protein levels in IGF-I-treated cells generally reflected the expression of ZO-1 mRNA (Fig. 4*B*).

Interactions of ZO-1 with the E-cad Complex in MCF-7/IGF-IR Cells—It has been recently reported that ZO-1 is an element of the E-cad complex (12–14). This complex also contains the IGF-IR, as described in our previous work (7, 8). Here, we analyzed tyrosine phosphorylation status of the IGF-IR, E-cad, and ZO-1, and the interactions among these proteins in MCF-7 and MCF-7/IGF-IR cells cultured as three-dimensional spheroids (Fig. 5). The autophosphorylation of the IGF-IR was elevated in MCF-7/IGF-IR cells, reflecting the increased responsiveness of the cells to IGF-IR ligands (IGF-I, IGF-II, and insulin) present in serum. However, tyrosine phosphorylation of E-cad and ZO-1 were unaffected by IGF-IR overexpression (Fig. 5*A*). Similarly, high levels of the IGF-IR did not affect tyrosine phosphorylation of α-, β-, or γ-catenin (data not shown). Next, we asked whether hyperactivation of the IGF-IR and increased expression ZO-1 have consequences for the associations among the proteins within the E-cad complex. Co-immunoprecipitation experiments demonstrated that IGF-IR overexpression resulted in an increased abundance of IGF-IR·E-cad and IGF-IR·ZO-1 complexes (Fig. 5*A*). Also, the elevated levels of ZO-1 in MCF-7/IGF-IR cells coincided with an increased association of ZO-1 with either E-cad or the IGF-IR (Fig. 5*A*). Moreover, the binding of α-catenin (a ZO-1-associated protein) to the IGF-IR or ZO-1 but not to E-cad was greater in MCF-7/IGF-IR cells than in MCF-7 cells (Fig. 5*A*). The presence of α-catenin in IGF-IR immunoprecipitates was confirmed with cell lysates in which α-catenin was first removed with a specific antibody. As expected, immunoprecipitation of such depleted lysates with either anti-IGF-IR or anti-E-cad antibodies revealed reduced α-catenin/E-cad and α-catenin/IGF-IR associations (Fig. 5*B*). Further experiments with α-catenin immunoprecipitates indicated increased abundance of α-catenin·actin and α-catenin·ZO-1 complexes in MCF-7/IGF-IR cells (Fig. 5*C*). A hypothetical model of possible interactions between adhesion proteins and the IGF-IR is shown in Fig. 6.





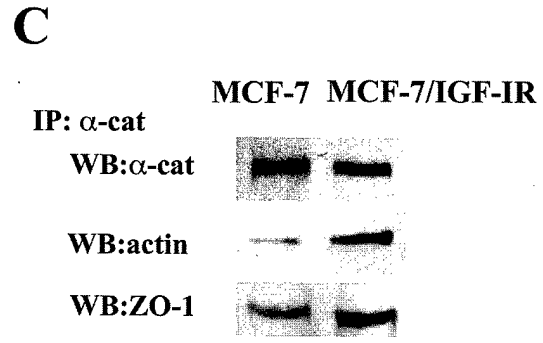


FIG. 5. **Interactions of ZO-1 with the IGF-IR in the E-cad complex.** *A*, the IGF-IR, E-cad, and ZO-1 were immunoprecipitated from 500 μg of total protein lysates obtained from MCF-7 and MCF-7/IGF-IR cells cultured as three-dimensional spheroids in normal growth medium. The immunoprecipitates (*IP*) were then probed by Western blotting (*WB*) for phosphotyrosine (*PY*), the IGF-IR (~97 kDa), ZO-1 (~220 kDa), E-cad (~120 kDa), and α-catenin (~102 kDa). *B*, to confirm α-catenin presence in IGF-IR immunoprecipitates, the lysates were first treated with anti-α-catenin antibodies (Zymed Laboratories Inc.) overnight to deplete α-catenin and then immunoprecipitated with either anti-E-cad or anti-IGF-IR antibodies. The E-cad and IGF-IR immunoprecipitates were then probed with another anti-α-catenin antibody (Sigma). Note significantly reduced α-catenin associations with IGF-IR and E-cad compared with that seen in *A*. *C*, 500 μg of protein lysates were precipitated with anti-α-catenin antibody and probed by WB for α-catenin, actin, and ZO-1. The blots presented in *A*, *B*, and *C* were identically developed with film exposure time 10 s.

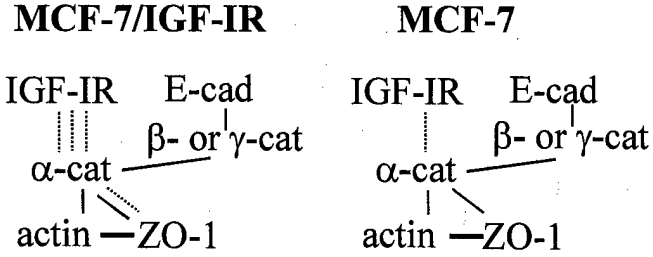


FIG. 6. **Possible interactions between ZO-1 and the IGF-IR within the E-cad complex.** The well established connections between E-cad, catenins, and actin are shown as *solid lines*. The proposed connections between the IGF-IR, α-catenin, ZO-1, and actin are drawn as *broken lines*. At present, it is not known whether the IGF-IR interacts with α-catenin directly or if other intermediate proteins are involved.

Down-regulation of ZO-1 Results in Decreased Cell-Cell Adhesion in MCF-7/IGF-IR Cells—Because the results suggested that ZO-1 may be an important intermediate in IGF-IR-stimulated cell-cell adhesion, we set out to confirm this notion using MCF-7/IGF-IR cells in which ZO-1 levels were down-regulated by the expression of an anti-ZO-1 RNA (MCF-7/IGF-IR/anti-ZO-1 cells) (Fig. 7). The clones with the best ZO-1 reduction and an intact E-cad and IGF-IR expression were analyzed in three-dimensional culture. The cell-cell adhesion of MCF-7/IGF-IR/anti-ZO-1 cells was greatly inhibited compared with that in the parental MCF-7/IGF-IR cells (Fig. 7 and Table 1). The expression of the anti-ZO-1 plasmid in the parental MCF-7 cells was toxic, and no viable clones were obtained. The transfection of the sense-ZO-1 vector had no effect on cell-cell adhesion (data not shown).

DISCUSSION

Cell-cell adhesion is a known factor modulating the motility of tumor cells and consequently impacting tumor metastasis (26). The regulation of this process by exogenous growth factors is still not well understood. In E-cad-positive breast cancer cells, the overexpression or activation of the IGF-IR has been shown to stimulate cell-cell adhesion and reduce cell spreading *in vitro* or in organ culture (7–10). The IGF-IR has also been found co-localized and co-precipitated with the E-cad adhesion complex (7, 8). The mechanism of IGF-IR-stimulated E-cad-dependent cell-cell adhesion is unknown and has been investigated in this work. We discovered the following observations. 1) IGF-IR overexpression increased aggregation in E-cad-positive cells but not in E-cad-negative cells. 2) High expression of both IGF-IR and E-cad markedly improved cell aggregation in E-cad-negative cells. 3) IGF-IR-dependent cell-cell adhesion in E-cad-positive cells did not affect the expression of E-cad or α-, β-, or γ-catenins but coincided with up-regulation of ZO-1. 4) ZO-1 expression was induced by IGF-I and required IGF-IR tyrosine kinase activity, and 5) high levels of ZO-1 coincided with an increased IGF-IR/α-catenin/ZO-1 binding and improved ZO-1/actin association, whereas down-regulation of ZO-1 by the expression of an anti-ZO-1 RNA inhibited IGF-IR-dependent cell-cell adhesion. We hypothesize that the mechanism or one of the mechanisms by which the activated IGF-IR stimulates cell-cell adhesion is overexpression of ZO-1 and the resultant stronger connections between the E-cad complex and the actin cytoskeleton.

Very little is known about the regulation of ZO-1 by growth

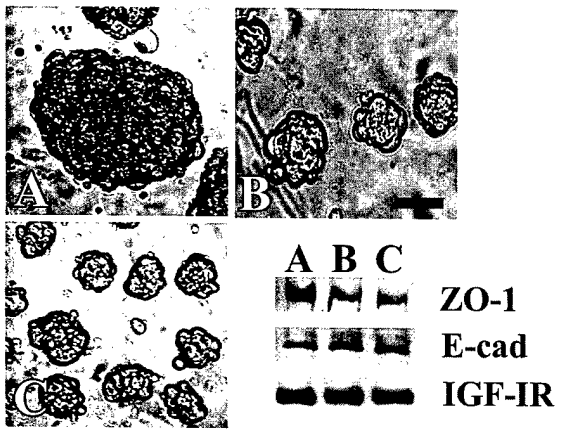


FIG. 7. **Reduced cell-cell adhesion in MCF-7/IGF-IR/anti-ZO-1 cells.** MCF-7/IGF-IR/anti-ZO-1 clones were obtained by stable transfection of MCF-7/IGF-IR cells with an anti-ZO-1 RNA expression plasmid (see "Materials and Methods"). The levels of ZO-1, IGF-IR, and E-cad in the parental MCF-7/IGF-IR cells (*A*) and in the clones (*B* and *C*) were studied by WB in 50 μg of protein lysate. The aggregation of cells was studied in three-dimensional culture as described under "Materials and Methods." The *bar* in *B* represents 50 μm.

factors. Several growth factors (*e.g.* epidermal growth factor and vascular endothelial growth factor) have been demonstrated to increase tyrosine phosphorylation of ZO-1 in different cellular model systems (18, 19). Hyperphosphorylation of ZO-1 usually coincides with its departure from tight junctions into the cytoplasm and with increased permeability (18, 19). In addition, v-Src-increased ZO-1 tyrosine phosphorylation has been linked to decreased cell-cell adhesion (27). IGF-I, on the other hand, has been shown to stabilize ZO-1 in tight junctions and to preserve the epithelial barrier in embryonic kidney cells and in pig thyrocytes (28, 29). However, the effects of IGF-I on ZO-1 expression and function in breast cancer cells have never been explored. Our findings provide the first evidence that the activation of the IGF-IR up-regulates ZO-1 mRNA and protein levels without affecting ZO-1 tyrosine phosphorylation. Consistent with the results obtained in other models, we noted increased adhesion in cells overexpressing ZO-1 and reduced aggregation in cells with down-regulated ZO-1 levels.

IGF-IR tyrosine phosphorylation was required for the stimulation of ZO-1 expression, inasmuch as the basal levels of ZO-1 were not increased in MCF-7/IGF-IR/Y3F cells expressing a dominant-negative, kinase-defective mutant of the IGF-IR. However, the putative IGF-I signaling pathways leading to ZO-1 expression have yet to be characterized. Our preliminary data with MCF-7/IRS-1 cells, in which the major IGF-IR/IRS-1/phosphatidylinositol 3-kinase growth/survival pathway is hyperactivated (1), suggested that this pathway is not involved in ZO-1 regulation (data not shown).

The clinical implications of IGF-induced and ZO-1-mediated cell-cell adhesion on tumor development and progression are unknown. Until now, the data from our and other laboratories suggest that in E-cad-positive breast cancer cells IGF-IR improves cell-cell adhesion and cell survival in three-dimensional culture but at the same time reduces cell spreading (1). Thus, one consequence of IGF-IR overexpression in breast cancer could be increased growth and survival of the primary tumor but reduced cell metastasis. This hypothesis is consistent with the observation that the IGF-IR is a good prognostic indicator for breast cancer, as tumors with good prognosis express much higher levels of the IGF-IR than tumors with bad prognosis (1, 30, 31). Notably, an independent study has shown that in breast tumors, E-cad and ZO-1 are co-expressed and are markers of a more differentiated phenotype (17). A formal analysis of the correlations between ZO-1 and the IGF-IR is underway in our laboratory and should help in clarifying the role of the IGF-IR in breast cancer progression.

Acknowledgments—Drs. J. M. Anderson and A. S. Fanning (Yale University, New Haven, CT) generously provided the pSKZO-1 plasmid encoding the human ZO-1 cDNA. The E-cad expression vector pBAT-EM2 was a gift from Drs. M. Takeichi (Kyoto University, Kyoto, Japan) and T. Yoneda (University of Texas, San Antonio, TX). The MCF-7/Y3F clone was developed by Dr. M. Guvakova (University of Pennsylvania, Philadelphia, PA).

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Role of the IGF-I Receptor in the Regulation of Cell–Cell Adhesion: Implications in Cancer Development and Progression

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The insulin-like growth factor-I receptor (IGF-IR) is a ubiquitous multifunctional tyrosine kinase that has an important role in normal cell growth and development. However, abnormal stimulation of IGF-IR signaling has been implicated in the development of different types of tumors. The strong antiapoptotic activity of IGF-IR has been recognized as critical in IGF-I-dependent tumorigenesis, however, the impact of other IGF-IR functions, such as regulation of cell–cell and cell–matrix adhesion are also increasingly acknowledged. Here, on the model of breast cancer cells, we discuss how IGF-IR-dependent regulation of intercellular adhesion may affect cell survival, resistance to antiestrogens, and invasion. J. Cell. Physiol. 194: 108–116, 2002. © 2002 Wiley-Liss, Inc.

The research of the past decade provided ample evidence that the insulin-like growth factor-I receptor (IGF-IR) regulates normal cell growth and development, but its abnormal stimulation can contribute to the development of different types of tumors. The strong antiapoptotic activity of IGF-IR has been recognized as critical in IGF-I-dependent tumorigenesis, however, the impact of other IGF-IR functions, such as regulation of cell–cell and cell–matrix adhesion are also increasingly acknowledged. Here, on the model of breast cancer cells, we discuss how IGF-IR-dependent regulation of intercellular adhesion may affect cell survival, resistance to antiestrogens, and invasion.

INSULIN-LIKE GROWTH FACTOR-I RECEPTOR (IGF-IR)

IGF-IR is an evolutionary conserved and ubiquitous transmembrane tyrosine kinase activated by IGF-I, IGF-II or insulin at hyperphysiological doses (Blakesley et al., 1999). IGF-IR is capable of regulating diverse biological processes such as proliferation, survival, transformation, differentiation, cell–cell, and cell–substrate interactions (reviewed in Baserga, 1999, 2000; Adams et al., 2000; Le Roith, 2000; O'Connor et al., 2000; Surmacz, 2000; Valentinis and Baserga, 2001). The functions of IGF-IR in a given cellular system rely on the induction of specific signaling pathways. Which pathways are induced depends on the number of activated IGF-IRs, availability of intracellular signal transducers, action of negative regulators, and is influenced by extracellular modulators (Surmacz, 2000). Activation of IGF-IR results in tyrosine phosphorylation of its cytoplasmic beta-subunit, followed by recruitment of IGF-IR substrates, of which the most notable are insulin receptor substrate 1 (IRS-1) and src- and collagen-homology (SHC) protein. IGF-IR-induced tyrosine phosphorylation of IRS-1 and SHC allows them to bind several effector proteins (enzymes and/or adapters) and activate multiple downstream signaling pathways (Shepherd et al., 1998; White, 1998; Adams et al., 2000; O'Connor et al., 2000; Surmacz, 2000; Burks and White, 2001).

A well-recognized and extensively studied function of IGF-IR is its antiapoptotic activity (Baserga, 1999, 2000; Blakesley et al., 1999; Le Roith, 2000; O'Connor et al., 2000; Valentinis and Baserga, 2001). IGF-IR can transmit survival signals through multiple, partially redundant, pathways. For instance, there are at least three pathways induced by IGF-IR all leading to the phosphorylation and inactivation of a pro-apoptotic protein BAD, namely, the IRS-1/PI-3K/Akt pathway, the SHC/Ras/ERK1/2 pathway, and mitochondrial translocation of Raf-1 (Peruzzi et al., 1999, 2001). In addition, stimulation of the Akt pathway by IGF-I represses the activity of GSK3-beta kinase leading to the accumulation of two GSK-3-beta targets: cyclin D1

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and beta-catenin (Ferkey and Kimelman, 2000; Desbois-Mouthon et al., 2001; Satyamoorthy et al., 2001; Yu et al., 2001). Higher levels of cyclin D1 result in the stabilization of the cyclin D1:cdk4 complex, hyperphosphorylation of Rb1 and stimulation of transcription of growth-related genes. The accumulation of beta-catenin, its translocation to the nucleus and binding to the T-cell factor (Tcf) transcriptional complex results in the activation of transcription of several growth/survival proteins, such as c-Myc, cyclin D1, and Id2 (Behrens, 1999, 2000; Novak and Dedhar, 1999; Rockman et al., 2001). Further positive effects of IGF-IR on cell cycle machinery include upregulation of cyclin E expression and increased activation of cyclin E:cdk2 complexes (Dupont et al., 2000). Other mitogenic/survival IGF-IR pathways operating in some cellular systems involve signal transducers and activators of transcription (STATs) (Zong et al., 1998, 2000; Prisco et al., 2001).

Importantly, several of the IGF-IR growth/survival pathways are engaged in a crosstalk with pathways induced by cell–cell or cell–matrix interactions, for instance SHC (Wary et al., 1996; Mauro et al., 1999), IRS-1 (Vuori and Ruoslahti, 1994; Reiss et al., 2001), IRS-2 (Shaw, 2001) associate with different integrin receptors and transmit matrix-dependent survival signals. Different elements of IGF-IR signaling system (IGF-IR, IRS-1, SHC) also interact with the molecules involved in the regulation of cell–cell adhesion, and IGF-I-dependent cell–cell adhesion has been shown to improve cell survival (Guvakova and Surmacz, 1997; Mareel et al., 1997; Surmacz et al., 1998; Surmacz, 2000; Valentinis et al., 1998; Mauro et al., 2001).

It is now quite evident that IGF-IR is an important factor in the development and progression of neoplastic processes. Numerous clinical and experimental data indicated that IGF-IR is involved in the tumorigenesis of breast (Surmacz, 2000), prostate (Djavan et al., 2001; Nickerson et al., 2001), pancreas (Korc, 1998), lung (Wu et al., 2000), colon (Giovannucci, 2001), liver (Scharf et al., 2001), ovary (Burroughs et al., 2002; Druckmann and Rohr, 2002), brain (Zumkeller and Schwab, 1999), and bladder (Hursting et al., 2001). The tumorigenic potential of IGF-IR usually relies on hyperactivation of IGF-I signaling. Generally, this hyperactivation is related to overabundance of the ligand, overexpression of the receptor, or upregulation of the PI-3K/Akt pathway resulting from overexpression of signaling substrates, such as IRS-1 and/or inactivation of PTEN phosphatase (Surmacz, 2000; Reiss et al., 2001; Burroughs et al., 2002; Nicholson and Anderson, 2002). Here we focus on one of the consequences of IGF-IR overexpression in breast cancer cells, i.e., increased cell–cell adhesion. We discuss the relevance of this phenomenon to cancer cell survival, antiestrogen-resistance, and invasive capacity.

CELL–CELL ADHESION AND THE E-CADHERIN COMPLEX

Cell–cell adhesion plays a critical role in normal organogenesis and differentiation but can also regulate processes such as proliferation, survival, migration, invasion (Gumbiner, 1996; Yamada and Geiger, 1997; Potter et al., 1999). Intercellular adhesion primarily involves direct homophilic interactions between cell-surface molecules such as the cadherins (Takeichi, 1990, Takeichi, 1991; Boggon et al., 2002). Cadherins are calcium-dependent cell–cell adhesion molecules that mediate homotypic interactions among cells and modulate tissue morphogenesis (Takeichi, 1991). The cadherin family includes the N-, P-, R-, B-, and E-cadherins as well as approximately 10 other members (Kemler, 1992; Takeichi, 1995; Adams, 1997). The structure of a typical classic cadherin consists of an amino-terminal external domain having five tandem repeats, a single transmembrane segment, and a cytoplasmic carboxy-terminal domain of approximately 150 amino acids. The binding functions of the cadherin are localized in the amino-terminal tandem repeat, whereas the other repeats are bridged by calcium binding sites that impart rigidity to the molecule (Aberle et al., 1996).

A well-studied functional element of adherens junctions is the adhesion complex organized around epithelial cadherin (E-cad) (Fig. 1). Several molecules associate with E-cad linking it to the actin cytoskeleton. The carboxy-terminal part of the cytoplasmic domain of E-cad associates with beta-catenin or plakoglobin (also called gamma catenin) whereas the juxtamembrane part binds to p120 catenin (Ozawa et al., 1990; Yap et al., 1998). Beta-catenin and plakoglobin directly interact with the cytoplasmic tail of E-cad but form mutually exclusive complexes (Hulsken et al., 1994). Via their amino-terminal domains both proteins associate with the vinculin-related protein alpha-catenin which in turn links to actin filaments (Herrenknecht et al., 1991; Nagafuchi et al., 1991; Geiger and Ayalon, 1992; Kemler, 1992; Hulsken et al., 1994; Watabe-Uchida et al., 1998; Weiss et al., 1998).

Reduced, absent, or altered expression of E-cad and/or catenins, and defective cell–cell connections have been shown to contribute to the development and progression of different tumors (Berx and Van Roy, 2001; Van Aken et al., 2001; Bremnes et al., 2002; Conacci-Sorrell et al., 2002; Hajra and Fearon, 2002). In breast cancer, invasive lobular carcinomas are typically completely E-cad-negative and often show inactivating mutations in E-cad gene. Ductal breast cancers in general show heterogeneous loss of E-cad expression, associated with epigenetic transcriptional downregulation. It has been suggested that the microenvironment at the invasive front can transiently downregulate E-cad transcription. This can be associated with induction of nonepithelial cadherins (Berx and Van Roy, 2001; Wheelock et al., 2001).

Several studies demonstrated that E-cad has tumor suppressor function. For instance, re-expression of E-cad in E-cad-negative mammary breast cancer cells induced cell–cell adhesion and reduced bone and lung metastasis (Takeichi, 1990; Frixen et al., 1991; Mareel et al., 1994; Mbalaviele et al., 1996; Meiners et al., 1998; Mauro et al., 2001). Similarly, the transgenic E-cad expression in nude mice blocked metastatic process, while the expression of a dominant negative E-cad promoted early invasion and metastasis in a pancreatic beta-cell carcinogenesis model (Perl et al., 1998). The inactivation of E-cad by specific antibodies results in the loss of cell–cell contact (Behrens et al., 1989; Mareel et al., 1995, 1996; Kandikonda et al., 1996; Guvakova

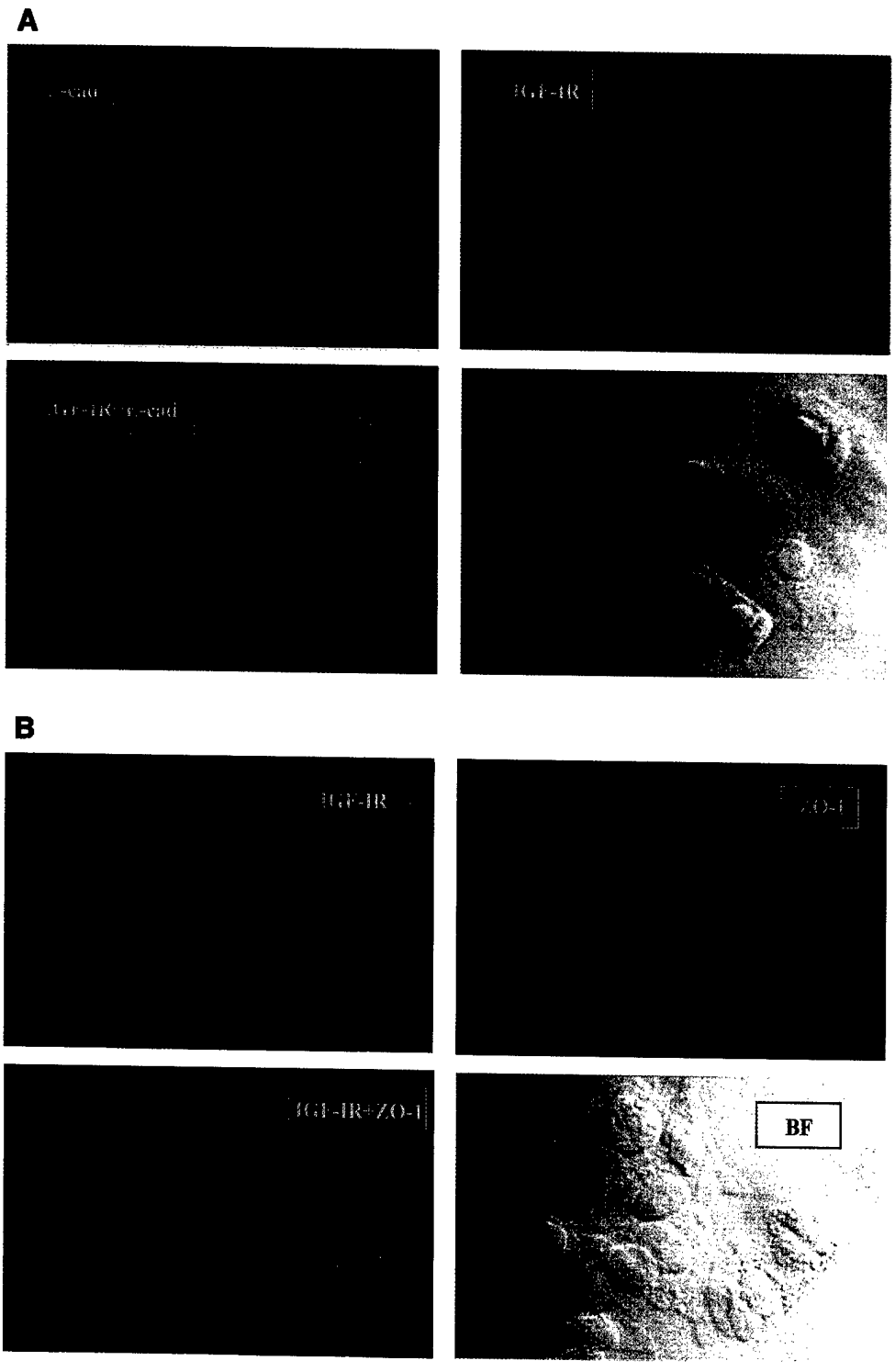

Fig. 1

and Surmacz, 1997). Decreased cell–cell connections have been associated with increased cell migration or decreased cell survival (Bracke et al., 1997; Mareel et al., 1997; Keirsebilck et al., 1998; Van Aken et al., 2001).

REGULATION OF THE E-CADHERIN COMPLEX

The function of the E-cad-complex can be regulated not only by the abundance of E-cad and catenins but also by posttranslational modifications of these proteins, most notably by tyrosine phosphorylation. For instance, the experiments of Daniel and Reynolds (1997) demonstrated that treatment of epithelial cells by orthovanadate, which blocks tyrosine phosphatase, leads to rapid and reversible loss of cell–cell contacts. The main target of tyrosine phosphorylation in the E-cad complex appears to be beta-catenin. Increased tyrosine phosphorylation of beta-catenin was observed in cells treated with epidermal growth factor, scatter factor/hepatocyte growth factor, transforming growth factor beta-1 as well as in v-Src-transfected cells; in all cases hyperphosphorylation of beta-catenin was associated with decreased intercellular adhesion and increased scattering (Matsuyoshi et al., 1992; Hazan and Norton, 1998; Noe et al., 1999; Hirohashi, 2000; Tian and Phillips, 2002). Importantly, several molecules regulating beta-catenin tyrosine phosphorylation, such as cytoplasmic and transmembrane tyrosine kinases c-Src and c-erbB-2 as well as tyrosine phosphatase LAR were localized to the E-cad-based adherens junctions (Hoschuetzky et al., 1994; Ochiai et al., 1994; Daniel and Reynolds, 1997; Behrens, 1999; Muller et al., 1999; Hirohashi, 2000; Symons et al., 2002).

IGF-IR MODULATES THE E-CADHERIN COMPLEX

The role of IGF-IR in the regulation of intercellular adhesion appears to be cell-specific. For instance, in C10 colorectal cancer cells, IGF-I-stimulated cell spreading, which was accompanied by tyrosine phosphorylation of beta-catenin, dissociation of beta-catenin from E-cad and translocation of beta-catenin from a sub-membrane to a cytoplasmic compartment (Playford et al., 2000). In addition, Andre' et al. (1999) demonstrated enhanced tyrosine phosphorylation of beta-catenin upon IGF-I stimulation in an APC-mutated colorectal cancer cell line, HT29. This resulted in down-regulation of E-cad and increased cell motility. Thus, in certain cell context, IGF-IR may inhibit the function of E-cad, which, could lead to a propensity for metastasis.

Unlike in C10 and HT29 cells, we found that the effects of IGF-I in E-cad-positive MCF-7 and MCF-7/6 breast cancer cell lines are related to stabilization rather than inhibition of cell–cell adhesion. Specifically, we demonstrated that IGF-I treatment, overexpression of IGF-IR and overexpression of IGF-I, all resulted in increased cell–cell adhesion (Bracke et al., 1993; Deman et al., 1995; Vermeulen et al., 1995; Guvakova and Surmacz, 1997; Surmacz et al., 1998; Mauro et al., 2001). Importantly, the effects of IGF-IR overexpression and IGF-I treatment on cell–cell adhesion were blocked with anti-E-cad antibody and were absent in E-cad-negative MDA-MB-231 breast cancer cells (Guvakova and Surmacz, 1997; Mauro et al., 2001).

IGF-IR not only modulates cell–cell adhesion, it also interacts with the E-cad complex. We found that IGF-IR co-localized and co-precipitated with E-cad, and some of the IGF-I signaling molecules are present in E-cad immunoprecipitates (Fig. 1A) (Guvakova and Surmacz, 1997; Surmacz et al., 1998; Mauro et al., 2001). The studies on molecular mechanisms of IGF-IR-dependent cell–cell adhesion indicated that IGF-IR overexpression or IGF-I treatment of MCF-7 cells did not alter the expression or tyrosine phosphorylation of E-cad or alpha-, beta-, or gamma-catenins (Guvakova and Surmacz, 1997; Surmacz et al., 1998; Mauro et al., 2001). Instead, IGF-I-increased cell–cell adhesion coincided with overexpression of zonula occludens protein-1 (ZO-1). ZO-1 is an ~220 kDa scaffolding protein containing various domains (as SH3 domain, three PDZ domains, a proline-rich region, and a guanylate kinase domain) that allow its interaction with specialized sites of plasma membrane as well as with other proteins (Willott et al., 1993; Tsukita et al., 1997). ZO-1 is a characteristic element of tight junctions, but recently its presence has also been demonstrated in E-cad adherens junctions (Rajasekaran et al., 1996; Itoh et al., 1997; Provost and Rimm, 1999; Mauro et al., 2001). The role of ZO-1 in adherens junctions is still unclear, but it is assumed that it may functionally link E-cad with the actin cytoskeleton because it associates with alpha-catenin and actin through its N- and C-terminus, respectively (Itoh et al., 1997; Mauro et al., 2001). In addition, as a member of the membrane-associated guanylate kinase homologue (MAGUK) family of putative signaling proteins, ZO-1 may be involved in signal transduction (Yamamoto et al., 1997).

In breast cancer, ZO-1 is usually co-expressed with E-cad and is a strong independent marker of a more differentiated phenotype (Hoover et al., 1998). We

Fig. 1. Co-localization of IGF-IR and E-cad (**A**) and IGF-IR and ZO-1 (**B**) in MCF-7/IGF-IR cells. A: MCF-7/IGF-IR clone 12 expressing ~250,000 IGF-IR/cell (Guvakova and Surmacz, 1997) were grown on coverslips in DMEM:F12 plus 5% calf serum (CS) to 50% confluence, then fixed in 3% paraformaldehyde, permeabilized with 0.2% Triton X-100, washed 3× with PBS, and incubated for 1 h with primary antibodies: anti-IGF-IR pAb (Santa Cruz Biotechnologies, Santa Cruz, CA) 10 μg/ml and anti-E-cad mAb (Santa Cruz) 2 μg/ml, then washed with PBS 3×, and incubated with secondary antibodies. The fluoresceine-conjugated donkey anti-mouse IgG was used as a secondary Ab for E-cad and rhodamine-conjugated donkey anti-rabbit IgG was used for IGF-IR. The IGF-IR and E-cad localizations were studied with Bio-Rad MRC 1024 confocal microscope connected to a Zeiss Axiovert 135M inverted microscope. The captured images of IGF-IR (red fluorescence), E-cad (green fluorescence), merged IGF-IR and E-cad (IGF-IR + E-cad) (yellow fluorescence), and bright field (BF) are shown. B: IGF-IR was detected with anti-IGF-IR mAb (Calbiochem) and fluoresceine-conjugated donkey anti-mouse IgG; ZO-1 was identified with anti-ZO-1 pAb 10 μg/ml (Zymed) and rhodamine-conjugated donkey anti-rabbit IgG. The captured images of IGF-IR (green fluorescence), ZO-1 (red fluorescence), merged IGF-IR and ZO-1 (IGF-IR+ZO-1) (yellow fluorescence), and bright field (BF) are shown. The optical sections in A and B were taken at the central plane. The fluorophores were imaged separately to ensure no excitation/emission wavelength overlap. The experiments were repeated three times with similar results, representative fields are shown.

found that ZO-1 was overexpressed in breast cancer cells overexpressing IGF-IR or treated with IGF-I. Furthermore, ZO-1 co-localized and co-immunoprecipitated with IGF-IR (Fig. 1B) (Mauro et al., 2001). High levels of ZO-1 coincided with increased abundance of ZO-1/IGF-IR, IGF-IR/alpha-catenin, and ZO-1/actin complexes. On the other hand, downregulation of ZO-1 by antisense RNA expression decreased IGF-IR-dependent cell–cell adhesion (Mauro et al., 2001). Thus, one of the mechanisms by which the activated IGF-IR regulates E-cad-mediated cell–cell adhesion is the overexpression of ZO-1 and the resulting stronger connection between the E-cad complex and the actin cytoskeleton (Mauro et al., 2001).

SOME CONSEQUENCES OF IGF-IR-INCREASED CELL–CELL ADHESION IN BREAST CANCER CELLS

In estrogen receptor (ER)-positive breast cancer, IGF-IR is significantly (up to 14-fold) overexpressed and hyperphosphorylated compared with normal mammary epithelium or benign tumors (Resnik et al., 1998; Surmacz, 2000). The overexpression of IGF-IR correlates with the resistance of tumor cells to anti-cancer treatments and tumor recurrence at the primary site (Surmacz, 2000).

It has been demonstrated that in MCF-7 breast cancer cells, overexpression of different elements of the IGF system, such as IGF-IR or IRS-1, results in increased responsiveness to IGF-I and, in consequence, provides breast cancer cells with growth advantage and reduced estrogen growth requirement (Lee and Yee, 1995; Surmacz et al., 1998). Antiestrogens have been shown to block estrogen-stimulated growth as well as interfere with IGF-IR-dependent proliferation of breast cancer cells in monolayer culture. In particular, we demonstrated that in MCF-7 cells, as well as in MCF-7 overexpressing IGF-IR or IRS-1, growth inhibition in monolayer culture was achieved by a long-term treatment (4 days) with the nonsteroidal antiestrogen 4-OH-Tamoxifen as well as with the pure antiestrogen ICI 182,780. The primary target of antiestrogen action in MCF-7 cells and MCF-7 derivatives was IRS-1 and its downstream signaling through the PI-3K pathway (Guvakova and Surmacz, 1997; Salerno et al., 1999).

We studied whether antiestrogen-sensitivity can be affected by cell–cell adhesion. To this end, MCF-7 cells and MCF-7 cells overexpressing IGF-IR (MCF-7/IGF-IR cells) were cultured in three-dimensional (3-D) culture. Under these conditions, MCF-7 cells form small (~50 μm in diameter) spheroids, while MCF-7/IGF-IR cells aggregate into large (~300 μm) spheroids (Guvakova and Surmacz, 1997; Mauro et al., 2001). Both MCF-7 and MCF-7/IGF-IR spheroids are viable for several days, and MCF-7/IGF-IR spheroids may even increase in size and cell number during culture. Characteristically, IGF-IR is constitutively activated in MCF-7/IGF-IR spheroid cultures (Mauro et al., 2001).

Our results with ICI 182,780 indicated that antiestrogen treatment reduced the survival of MCF-7 spheroids by ~50%, but had no effect on MCF-7/IGF-IR spheroids (Table 1). However, when MCF-7/IGF-IR aggregates were disrupted with EGTA or anti-E-cad antibody, ICI 182,780 was able to reduce the number of cell. This suggested that IGF-IR-dependent cell–cell adhesion may represent a mechanism of enhanced resistance to antiestrogen treatment. It has recently been suggested that N-cadherin-mediated survival involves stimulation of the Akt pathway and upregulation of antiapoptotic protein Bcl2 or inactivation of proapoptotic protein BAD (Li et al., 2001; Tran et al., 2002). Our preliminary results indicated that despite higher tyrosine phosphorylation of IGF-IR in MCF-7/IGF-IR spheroids, in both cell models, the Akt pathway was activated to a similar extent under basal conditions and was comparably inhibited by ICI 182,780 treatment. Thus, the mechanism of antiestrogen resistance in MCF-7/IGF-IR spheroids may rely on a pathway distinct from the classic survival Akt pathway. The nature of this signal is under investigation in our laboratories.

Next, we probed the effect of IGF-IR-induced cell–cell adhesion on the invasive potential of breast cancer cells. We used MCF-7/6 cells that are characterized by a moderate invasive potential. The cells contain IGF-IR and express several elements of the E-cad complex (Bracke et al., 1993; Mareel et al., 1997). We previously demonstrated that IGF-I treatment decreased invasiveness of these cells through an E-cad-dependent mechanism (Bracke et al., 1993; Mareel et al., 1997). Using precultured chick heart fragments (PHF) we investigated whether overexpression of IGF-IR in MCF-7/6 cells modifies invasiveness (Bracke et al., 1993; Mareel et al., 1997). Two MCF-7/6/IGF-IR clones A6 and A9, obtained by stable transfection of IGF-IR cDNA, were studied. The cells were first aggregated and then cultured in contact with PHF for several days. The degree of breast cancer cell invasion into PHF was evaluated by histology. As shown in Table 2 and Figure 2, overexpression of IGF-IR significantly decreased invasiveness of MCF-7/6 cells bringing it to the levels comparable

TABLE 1. Antiestrogen resistance in cells grown in 3-D culture

Cell line	Control cell number ($\times 10^5$)	ICI cell number ($\times 10^5$)	Viability (%)	ICI + IGF-I cell number ($\times 10^5$)	Viability (%)
MCF-7	3.0 ± 0.0	1.4 ± 0.1	47	1.8 ± 0.2	57
MCF-7/IGF-IR	3.2 ± 0.2	3.0 ± 0.1	94	3.7 ± 0.1	>100
MCF-7/IGF-IR + anti-E-cad Ab	2.2 ± 0.1	1.3 ± 0.2	59	1.3 ± 0.2	59
MCF-7/IGF-IR + EGTA	2.0 ± 0.0	0.7 ± 0.1	35	1.1 ± 0.0	55

MCF-7 and MCF-7/IGF-IR cells were synchronized in phenol red-free serum-free medium (PRF-SFM) (Guvakova and Surmacz, 1997), trypsinized, washed in PRF-SFM and counted. Cells ($3–4 \times 10^5$) were plated into 60-mm plates coated with 2% agarose (to prevent cell-substrate adhesion). Formation of spheroids was induced by gentle rotation of cells on a shaker for the first 4 h of incubation at 37°C. After 24 h, the spheroids were treated for 4 days with 100 nM ICI 182,780 in the presence or absence of 50 ng/ml IGF-I. After that, the medium containing clustered cells was collected and the cells, dispersed through a G-21 needle and counted (with trypan blue to exclude dead cells). To assess the role of cell–cell adhesion, MCF-7/IGF-IR cells were incubated with either 3 mM EGTA or 2 mg/ml anti-E-cad blocking Ab in parallel with ICI 182,780 treatment. The number of cells recovered after 4 days of treatment is shown. Control, the number of ICI 182,780 untreated cells at day 4. Results are average from several experiments ± SE.

TABLE 2. Invasive properties of MCF-7/6/IGF-IR cells

Cell line	Day	Degree of invasion	Cell line	Day	Degree of invasion
MCF-7/6	4	III	MCF-7/A6/IGF-IR, A6	4	I
MCF-7/6	4	II	MCF-7/A6/IGF-IR, A6	4	I
MCF-7/6	4	II–III	MCF-7/A6/IGF-IR, A6	4	Lost
MCF-7/6	7	III	MCF-7/A6/IGF-IR, A6	7	I
MCF-7/6	7	III	MCF-7/A6/IGF-IR, A6	7	I
MCF-7/6	7	III	MCF-7/A6/IGF-IR, A6	7	I–II
MCF-7/AZ	4	I	MCF-7/A6/IGF-IR, A9	4	I
MCF-7/AZ	4	I	MCF-7/A6/IGF-IR, A9	4	I
MCF-7/AZ	4	I	MCF-7/A6/IGF-IR, A9	4	I
MCF-7/AZ	7	I	MCF-7/A6/IGF-IR, A9	7	II
MCF-7/AZ	7	I	MCF-7/A6/IGF-IR, A9	7	I
MCF-7/AZ	7	I	MCF-7/A6/IGF-IR, A9	7	I

Invasiveness of MCF-7/6/IGF-IR cells was tested using confronting organ culture with precultured chick heart fragments (PHF). The cells were studied in parallel with the parental cell line MCF-7/6 (invasive) and control MCF-7/AZ cells (non-invasive). Briefly, 9-day-old embryonic chick heart fragments were precultured and selected for diameter 0.4 mm. The heart fragments were confronted with cell aggregates of ~0.5 mm in diameter and cultured for 7 days. After fixation, the heart fragments were embedded in paraffin, serially sectioned and stained with hematoxylin-eosin. The degree of invasion of epithelial cell into heart was determined histologically (Bracke et al., 2001). Grade I: The confronting cells are attached to the PHF, and do not occupy the heart tissue, even not the outermost fibroblastic cell layers. Grade II: Marginal invasion, limited to the outer fibroblast-like and myoblast cell layers. Grade III: The confronting cells occupy the PHF, but have left more than half of the original heart tissue intact.

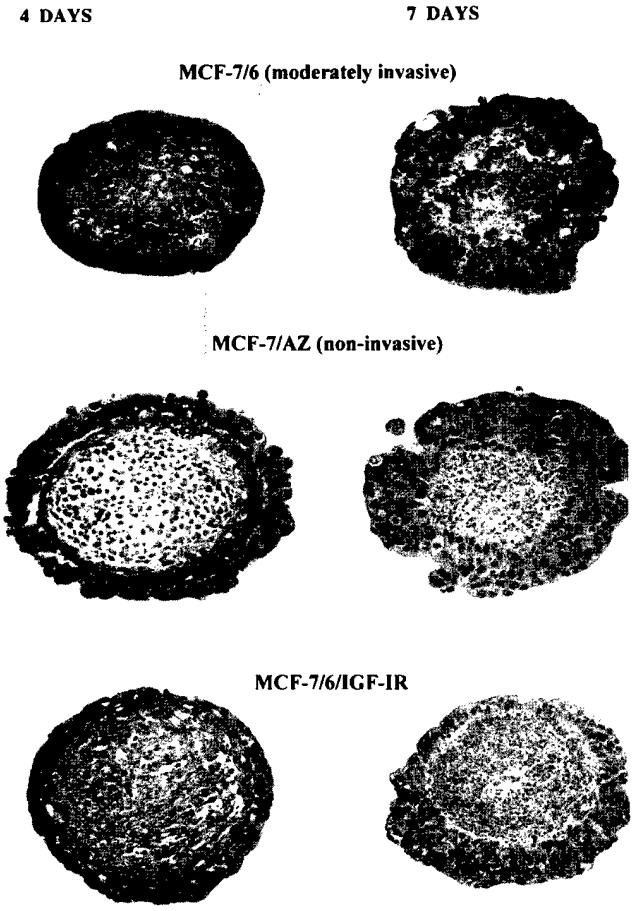

Fig. 2. Effects of IGF-IR overexpression on breast cancer cell invasion. MCF-7/6, MCF-7/6/IGF-IR, and MCF-7/AZ cells were tested as described under Table 2. The cultures were processed for histology (Bracke et al., 2001). Representative images are shown.

to that seen in MCF-7/AZ cells (non-invasive control, similar to MCF-7 cells). Notably, overexpression of IGF-IR in E-cad-negative MDA-MB-231 breast cancer cells had no effect on their invasive potential.

In summary, IGF-IR seems to play an important role in the regulation of intercellular adhesion, but its effects are cell-specific. Our studies with breast cancer model indicate that in E-cad positive breast cancer cells, IGF-IR-dependent adhesion may contribute to cell survival and antiestrogen resistance in primary tumors; on the other hand, increased cell–cell adhesion may produce anti-metastatic effects (Guvakova and Surmacz, 1997; Surmacz, 2000; Mauro et al., 2001). These observations agree with the clinical data that in ER-positive primary breast cancer, IGF-IR is overexpressed and promotes tumor recurrence and resistance to anti-cancer treatments. Notably, E-cad as well as IGF-IR are usually co-expressed with ER (Guvakova and Surmacz, 1997; Surmacz et al., 1998; Berns et al., 1992). The observation that overexpression of IGF-IR may reduce metastatic potential in E-cad-positive cells concurs with the clinical observation that metastatic breast cancer cells express low levels of both IGF-IR and E-cad and that low IGF-IR levels correlate with bad prognosis in breast cancer (Peyrat and Bonneterre, 1992; Railo et al., 1994; Siitonen et al., 1996; Lee et al., 1999; Schnarr et al., 2000).

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Estrogen receptor-α regulates the degradation of insulin receptor substrates 1 and 2 in breast cancer cells

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In breast cancer cells, 17-β-estradiol (E2) upregulates the expression of insulin receptor substrate 1 (IRS-1), a molecule transmitting insulin-like growth factor-I (IGF-I) signals through the PI-3K/Akt survival pathways. The stimulation of IRS-1 by E2 has been documented on the transcriptional level. Here we studied whether the expression of estrogen receptor (ER)-α affects IRS molecules post-transcriptionally. We used ER-α-negative MDA-MB-231 breast cancer cells and MDA-MB-231 cells with re-expressed ER-α. In MDA-MB-231 cells cultured under serum-free conditions, IRS-1 and IRS-2 were degraded through the 26S proteasome and calpain pathways. Re-expression of ER-α in MDA-MB-231 cells correlated with enhanced stability of IRS molecules. This effect coincided with significantly reduced ubiquitination of IRS-1 and IRS-2, but did not involve increased IRS-1 and IRS-2 transcription. The interference of ER-α with IRS-1 and IRS-2 turnover could rely on the competition for common degradation pathways, as in MDA-MB-231/ER cells, ER-α processing was blocked by proteasome and calpain inhibitors. Notably, a fraction of the cytosolic ER-α colocalized and coprecipitated with IRS-1 and IRS-2, indicating a possible common destination for these proteins. The stabilization of IRS-1 in MDA-MB-231/ER cells was paralleled by the upregulation of the IRS-1/Akt/GSK-3 pathway and improved survival in the presence of IGF-I, whereas IRS-2 was not involved in IGF-I signaling.
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Introduction

Insulin receptor substrates (IRS) 1 and 2, members of the IRS family of signaling molecules, are major signaling intermediates of the insulin and insulin-like growth factor I (IGF-I) receptors (IR and IGF-IR). In addition, IRS-1 and IRS-2 transmit signals of many other receptors (e.g., prolactin, growth hormone, several interleukins, and interferons, α6β4 integrins) (Yenush and White, 1997; Aguirre and White, 2000; Burks and White, 2001; Shaw, 2001). In response to ligand binding, IRS substrates are tyrosine phosphorylated, which results in sequestration of multiple effector molecules and stimulation of different signaling pathways (Yenush and White, 1997; Aguirre and White, 2000; Burks and White, 2001). The most notable is the PI-3K/Akt pathway that regulates cell growth and survival as well as different nonmitogenic functions in various cell systems (Shepherd *et al.*, 1998) Although the functions of IRS-1 and IRS-2 can partially overlap, the data obtained with knockout animals indicated that IRS-1 and IRS-2 may have unique roles (Burks and White, 2001; Fasshauer *et al.*, 2001).

Aberrant expression of IRS molecules has been associated with pathogenesis of diabetes as well as with the development of cancer of the breast, pancreas, and liver (Sasaki *et al.*, 1993; Bergmann *et al.*, 1996; Kornmann *et al.*, 1998; Spector *et al.*, 1999; Aguirre and White, 2000; Surmacz, 2000; Ducluzeau *et al.*, 2001; Sachdev and Yee, 2001). However, the mechanisms controlling cellular abundance of these substrates are only partially known. Recent studies indicated that the expression of IRS-1 and IRS-2 is developmentally regulated and cell context-dependent, and may involve transcriptional and post-transcriptional events (Giovannone *et al.*, 2000). For instance, 17-β-estradiol (E2) has been shown to increase IRS-1 mRNA expression, while antiestrogens decrease IRS-1 mRNA levels (Guvakova and Surmacz, 1997; Lee *et al.*, 1999; Salerno *et al.*, 1999; Molloy *et al.*, 2000; Surmacz, 2000; Mauro *et al.*, 2001; Sachdev and Yee, 2001). Glucocorticoids inhibit IRS-1, but stimulate IRS-2 mRNA expression (Turnbow *et al.*, 1994; Dupont *et al.*, 1999; Sakoda *et al.*, 2000). Furthermore, IRS-2 transcription can be induced by progesterone and PPAR gamma agonists and inhibited by insulin (Vassen *et al.*, 1999; Smith *et al.*, 2001; Zhang *et al.*, 2001).

In addition to the transcriptional control, cellular abundance of IRS proteins is regulated by post-transcriptional mechanisms, especially those controlling protein processing. For instance, in 3T3-L1 adipocytes, chronic exposure to insulin increased IRS-1 serine/

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threonine phosphorylation and resulted in IRS-1 degradation through the 26S proteasome (Sun *et al.*, 1999; Pederson *et al.*, 2001). Similarly, prolonged exposure of MCF-7 breast cancer cells and prostate epithelial cells to IGF-I increased IRS-1 ubiquitination and subsequent degradation in a 26S proteasome-dependent manner (Lee *et al.*, 2000; Zhang *et al.*, 2000). Other study with 3T3-L1 cells implicated calpain, a calcium-dependent neutral protease, in the degradation of IRS-1 in response to chronic insulin treatment (Smith *et al.*, 1993, 1996). The lysosomal pathway has been reported as not involved in IRS-1 processing (Rice *et al.*, 1993).

IRS-2 has been described to undergo degradation through the 26S proteasome in 3T3-L1 cells, Fao hepatoma cells, mouse embryo fibroblasts (Rui *et al.*, 2001) and mouse uterine cells (Richards *et al.*, 2001). However, in CHO cells, prolonged insulin exposure did not affect IRS-2 stability, while it resulted in proteasomal processing of IRS-1 (Sun *et al.*, 1999).

The proliferation and survival of hormone-responsive breast cancer cells is influenced by crosstalk between the estrogen receptor-α (ER-α) and IGF-IR. An important feature of this interplay is upregulation of IGF-IR signaling by activated ER-α (Surmacz, 2000; Sachdev and Yee, 2001). Especially, E2 has been shown to increase transcription and expression of IRS-1, potentiating the IRS-1/PI-3K/Akt pathway (Lee *et al.*, 1999; Surmacz, 2000; Sachdev and Yee, 2001). The regulation of IRS molecules by ER-α on the post-transcriptional level, especially on the level of protein degradation, has not been studied and is a subject of this work. Considering that the 26S proteasome and calpain pathways have been implicated in the degradation of ER-α (Murayama *et al.*, 1984; Shiba *et al.*, 1996; Nawaz *et al.*, 1999; Lonard *et al.*, 2000), we explored the idea that ER-α and IRS substrates may compete for the same degradation processes. To better distinguish between the transcriptional and post-transcriptional regulation of IRS-1 and IRS-2 by ER-α, we developed MDA-MB-231/ER breast cancer cells, in which ER-α does not affect IRS-1 and IRS-2 mRNA synthesis. The degradation of IRS-1 and IRS-2 was studied in MDA-MB-231/ER cells and compared to that in ER-α-negative MDA-MB-231 cells.

Results

Re-expression of ER-α in MDA-MB-231 cells coincides with decreased degradation of IRS-1 and IRS-2

MDA-MB-231 cells are ER-α-negative breast cancer cells expressing IRS-1 and IRS-2. To investigate the expression of IRS substrates in the presence or absence of ER-α, we reintroduced ER-α into MDA-MB-231 cells by stable transfection. Several clones positive for ER-α expression were pooled to generate a mixed population of MDA-MB-231/ER cells. In growing MDA-MB-231/ER cells (day 0), the levels of IRS-1 and IRS-2 were greater than that in the parental ER-α-negative cells (Figure 1a) During cell culture in SFM, the abundance of IRS-1 and IRS-2 in MDA-MB-231 cells further declined, reaching minimal levels at day 3, when the

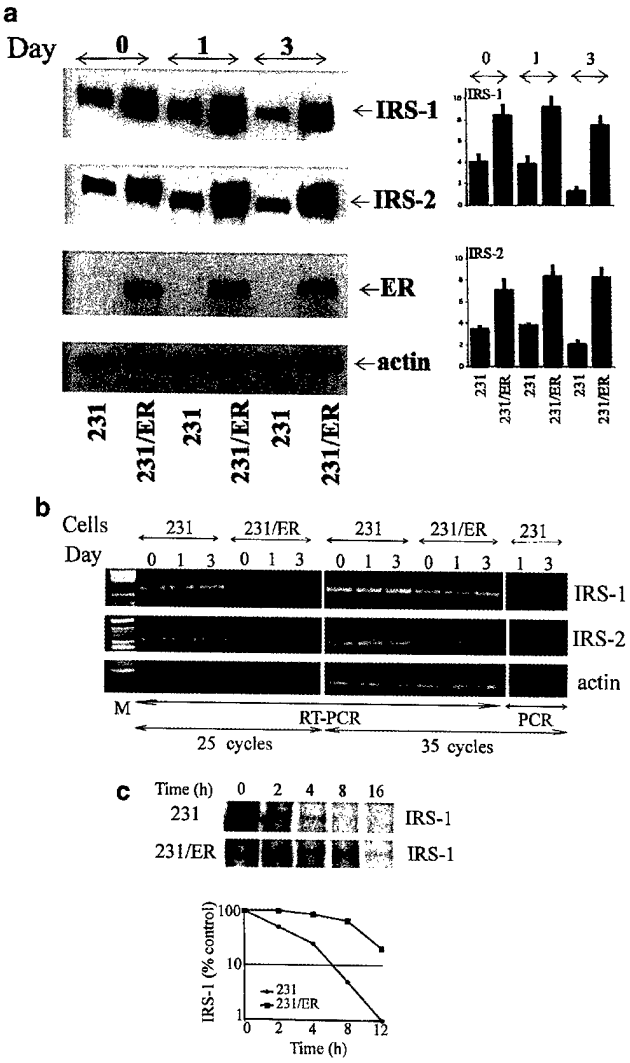

Figure 1 Expression of ER-α in MDA-MB-231 cells prevents degradation of IRS-1 and IRS-2. (**a**) Confluent cultures (70%) of MDA-MB-231 (231) and MDA-MB-231/ER (231/ER) cells were shifted to SFM for 0 (media change), 1, and 3 days. At indicated times, the cells were lysed and 50 μg of protein lysates were probed by WB for the expression of IRS-1, IRS-2, ER-α (ER), and β-actin (actin), as described in Materials and methods. The statistical variation of IRS-1 and IRS-2 protein expression is shown in the graph (bars, SE). (**b**) Total cellular RNA was isolated from MDA-MB-231 and MDA-MB-231/ER cells cultured in SFM for 0, 1, and 3 days. The expression of IRS-1, IRS-2, and β-actin (control of RNA input) mRNA was evaluated by RT–PCR as described in Materials and methods. The PCR products corresponding to IRS-1, IRS-2, and β-actin cDNA fragments (762, 381, and 209 bp, respectively) were obtained at 25 and 35 PCR cycles. To control the purity of RNA, the RT step was omitted before the samples were amplified by PCR. The experiments were repeated three times with the same result. (**c**) The half-life of IRS-1 was determined in MDA-MB-231 and MDA-MB-231/ER cells by pulse-chase with ^{35}S, as described in Materials and methods. IRS-1 abundance was analysed at 0, 2, 4, 8, and 12 h in cells cultured in SFM. The results, expressed as percentage control (IRS-1 levels at time 0) are represented in the graph

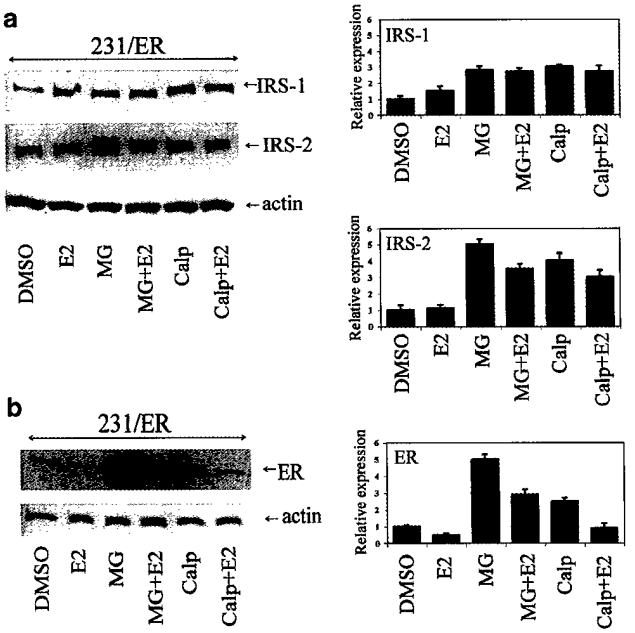

Figure 2 Degradation pathways of IRS-1, IRS-2, and ER-α in MDA-MB-231/ER cells. MDA-MB-231/ER cells were treated with E2, MG 132 (MG), MG 132 plus E2, calpastatin (Calp), and calpastatin plus E2 as described in Materials and methods. Following the treatment, the cells were lysed and 25 μg of total protein lysates were analysed by WB with specific Abs to detect IRS-1 and IRS-2 (**a**), and ER-α (**b**). To control for protein loading, the blots in (**a**) and (**b**) were stripped and re-probed with anti-β-actin Ab. The graphs represent the expression of each protein (±s.e.) relative to the expression of actin measured by laser scanning. The experiments were repeated four times

expression of IRS-1 and IRS-2 relative to the basal levels decreased by ~2.3- and 2.0-fold, respectively. In contrast, in MDA-MB-231/ER cultured in SFM, the levels of IRS-1 and IRS-2 remained nearly unchanged (Figure 1a).

To investigate whether the increased abundance of IRS-1 and IRS-2 in the presence of ER-α reflected increased transcription, the expression of IRS-1 and IRS-2 mRNAs was assessed by RT–PCR at days 0, 1, and 3. At all time points, the abundance of the PCR products obtained at 25 and 35 cycles was similar in MDA-MB-231 and MDA-MB-231/ER cells, indicating that re-expression of ER-α did not affect IRS-1 and IRS-2 on the transcriptional level (Figure 1b).

Consequently, we explored the possibility that the presence of ER-α might affect the stability of IRS-1 and IRS-2. We measured the half-life of the IRS molecules by ^{35}S pulse-chase labeling. Under SFM conditions, the half-life of IRS-1 in MDA-MB-231 cells was ~3 h, while in MDA-MB-231/ER cells ~10 h (Figure 1c) ER-α increased the half-life of IRS-2 by ~6 h in our cell models (data not shown).

Degradation of IRS-1, IRS-2, and ER occurs through the 26S proteasome and calpain pathways

To study the degradation pathways of IRS-1 and IRS-2 in MDA-MB-231/ER cells, we used specific blockers of the proteasome and calpain pathways. Treatment of the cells with either 10 μM MG 132, a 26S proteasome inhibitor, or 20 nM calpastatin, a calpain inhibitor, resulted in the accumulation of IRS-1 and IRS-2, indicating that these two pathways are involved in the processing of the IRS substrates. In the case of IRS-1, both MG 132 and calpastatin increased IRS-1 levels by approximately 2.8- and 3.0-fold, respectively (Figure 2a). IRS-2 levels were enhanced by ~5.0-fold in the presence of MG 132 and by ~4.0-fold in calpastatin-treated cells. The addition of 10 nM E2 did not affect the expression of IRS-1 in a significant way, while it reduced slightly the amounts of IRS-2 in MG 132 and calpastatin-treated cells (Figure 2a).

The inhibitors of the 26S proteasome and calpain were also employed to assess ER-α degradation. In the presence of MG 132, ER-α levels were significantly (~5.0-fold) increased, while calpastatin treatment resulted in 2.5-fold upregulation of ER-α expression (Figure 2b). Concurrent with published observations (Nawaz *et al*., 1999), stimulation with E2 significantly reduced (by ~50%) ER-α levels. Interestingly, this effect of E2 on ER-α was also evident in MG 132 and calpastatin-treated cells (Figure 2b). The observation that the inhibition of individual proteolytic processes was not sufficient to abolish E2 action suggested that multiple pathways are involved in E2-dependent degradation of ER-α. None of the treatments affected the expression of β-actin in MDA-MB-231/ER cells (Figure 2a and b).

Ubiquitination of IRS-1 and IRS-2 is significantly reduced in the presence of ER-α

Ubiquitination of proteins marks them for the recognition by the 26S proteasome (Joazeiro and Hunter, 2000). Treatment with MG 132, which blocks the 26S proteasome activity, results in the accumulation of ubiquitinated proteins. We analysed the ubiquitination of IRS-1, IRS-2, and ER-α in the presence or absence of MG 132 in MDA-MB-231 and MDA-MB-231/ER cells (Figure 3a, b, and c). In ER-α-negative MDA-MB-231 cells, IRS-1 and IRS-2 were ubiquitinated in MG 132-treated cells, and the addition of E2 did not affect this process. In contrast, in MDA-MB-231/ER cells, the ubiquitination of the IRS proteins in the presence of MG 132 was dramatically decreased with or without E2 (Figure 3a and b). The results suggested that in the presence of ER-α, the proteasome-dependent degradation of IRS-1 and IRS-2 might be inhibited at the step of ubiquitination. This could occur if the elements of the ubiquitination cascade involved in IRS-1 and IRS-2 processing were sequestered by re-expressed ER-α. Subsequent experiments indicated that ER-α was ubiquitinated in MG 132-treated MDA-MB-231/ER cells (Figure 3c). In the presence of E2, the ubiquitination was reduced, most likely reflecting ligand-induced downregulation of ER-α expression (Figures 3c and 2b).

The effect of ER-α expression on the ubiquitination of IRS-1 and IRS-2 was at least partially specific, as it did

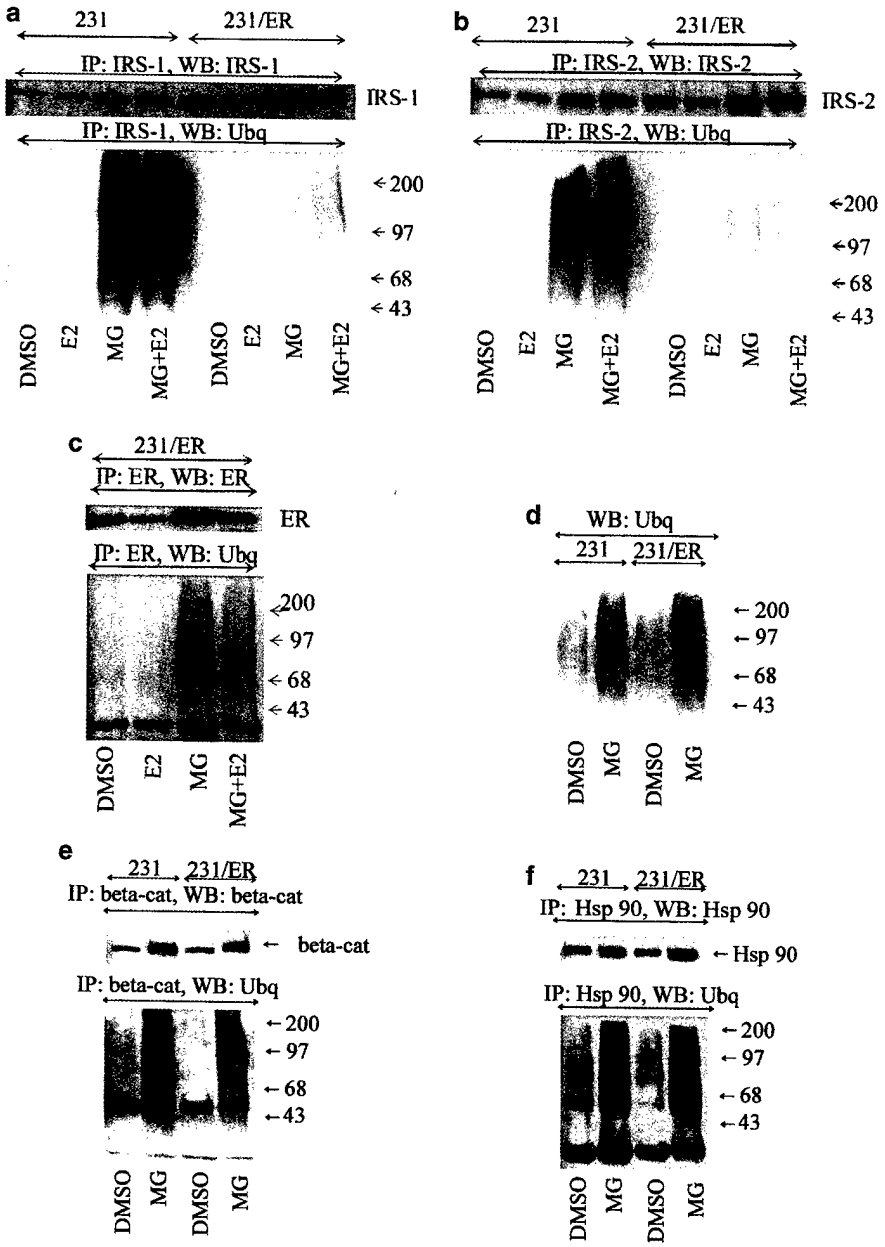

Figure 3 ER-α expression coincides with reduced ubiquitination of IRS-1 and IRS-2 in MDA-MB-231/ER cells. MDA-MB-231 and MDA-MB-231/ER cells were treated for 1 day with either E2 (10 nM), MG 132 (10 μM), E2 plus MG 132, or DMSO (7 mM) (control treatment) and then lysed. IRS-1 (**a**), IRS-2 (**b**), or ER-α (**c**) were immunoprecipitated from 1 mg of protein lysates, and their levels and ubiquitination were evaluated by WB with specific Abs, as described in Materials and methods. The ubiquitination of total cytoplasmic proteins (**d**) was studied by WB in 50 μg of protein lysates obtained from MDA-MB-231 and MDA-MB-231/ER cells treated with MG 132 (10 μM) or DMSO (7 mM). The same lysates (500 μg) were used to immunoprecipitate β-catenin (beta-cat) (**e**) and Hsp 90 (**f**) and analyse their ubiquitination by WB with appropriate Abs. The data are representative of at least three experiments.

not reduce total protein ubiquitination, or the ubiquitination of other proteins that are normally degraded through proteasome-dependent pathways (Figure 3d, e, and f). For instance, the ubiquitination of β-catenin and heat shock protein 90 (Hsp 90), both targets of the 26S proteasome (Aberle *et al*., 1997; Ashok *et al*., 2001), were comparable in MDA-MB-231 and MDA-MB-231/ER cells ER (Figure 3e and f). Similarly, ER-α did not affect ubiquitination of Hsp 70 and IRS-4 (data not shown).

IRS-1 and IRS-2 colocalize and coprecipitate with ER-α

Since ER-α appeared to affect the processing of IRS-1 and IRS-2, we studied whether all these proteins reside within the same cellular compartment. Using confocal microscopy and subcellular fractionation, we found that in untreated MDA-MB-231/ER cells, ER-α is present in the cytoplasm as well as in the nucleus, while IRS-1 and IRS-2 were mostly cytoplasmatic (Figure 4a and b, and unpublished data). Small amounts of IRS-1 and IRS-2

were present in the nucleus of MDA-MB-231/ER cells, while nuclear IRS-1 and IRS-2 were undetectable in MDA-MB-231 cells (Figure 4b), confirming recent reports that IRS molecules can translocate to the nuclear compartment when coexpressed with nuclear proteins (Lassak *et al.*, 2002; Prisco *et al.*, 2002). In MDA-MB-231/ER cells, a significant fraction (~30%) of IRS-1 colocalized with ER-α in the cytoplasm (Figure 4a). Similar colocalization was found for IRS-2 and ER-α (data not shown).

The subsequent experiments demonstrated that in MDA-MB-231/ER cells, ER-α can be found in IRS-1 and IRS-2 immunoprecipitates, while it cannot be detected in IRS-1 or IRS-2 precipitates from ER-α-negative MDA-MB-231 cells (Figure 4c). In a complementary experiment, we located IRS-1 and IRS-2 in ER-α precipitates in MDA-MB-231/ER cells (Figure 4d). Consistent with our previous findings (Figures 2 and 3), the abundance of ER-α : IRS-1 and ER-α : IRS-2 complexes was always significantly greater in MG 132-treated cells, and reflected increased accumulation of IRS-1, IRS-2, and ER-α in the presence of the proteasome 26S inhibitor (Figure 4c and d). To confirm that a fraction of ER-α can associate with IRS-1 and IRS-2, depletion experiments were performed, where the expression of ER-α was probed in cytosolic lysates before and after IRS-1 or IRS-2 precipitation. We found that depletion of IRS-1 and IRS-2 significantly (~35 and ~25%, respectively) reduced cytoplasmic abundance of ER-α (Figure 4e).

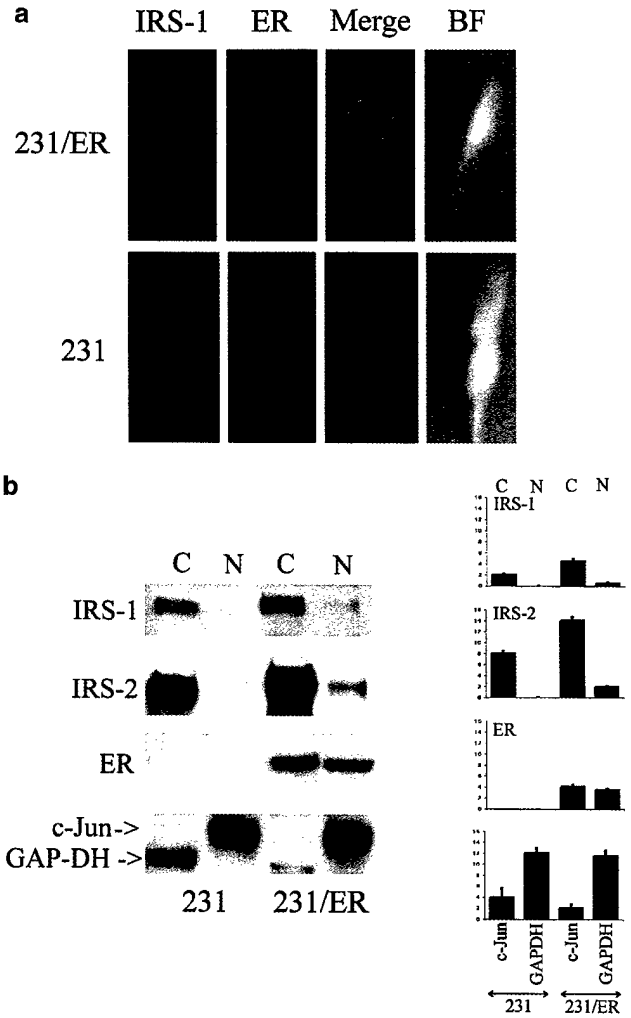

Figure 4 IRS-1 and IRS-2 colocalize and coprecipitate with ER-α in MDA-MB-231/ER cells. (**a**) The localization of IRS-1 and ER-α in unstimulated MDA-MB-231/ER and MDA-MB-231 cells was studied by confocal microscopy, as described in Materials and methods. The captured images of IRS-1 (red fluorescence), ER-α (green fluorescence), merged IRS-1 and ER-α (Merge) (yellow fluorescence), and bright field (BF) are shown. The representative images of several experiments are shown. (**b**) The expression of IRS-1, IRS-2, and ER-α was studied in 50 μg of cytoplasmic (C) and nuclear (N) protein lysates obtained from MDA-MB-231 (231) and MDA-MB-231/ER (231/ER) cells. The expression of a nuclear protein, c-Jun, and a cytoplasmic enzyme, GAP-DH, was assessed (upon stripping and reprobing of the filters) to control the purity of fractions. The graph represents relative abundance of each protein (±SE). (**c**) MDA-MB-231 and MDA-MB-231/ER cells were treated for 1 day with 10 μm MG 132 or 7 mM DMSO and then lysed. IRS-1 and IRS-2 were immunoprecipitated from 1 mg of protein lysates and their association with ER-α was probed by WB with anti-ER-α mAb. In parallel, the expression of ER-α in the cell lines was studied by WB in 50 μg of whole protein lysates. (**d**) ER-α was immunoprecipitated from 1 mg of protein lysates and its association with IRS-1 and IRS-2 was evaluated by WB with anti-IRS-1 and -IRS-2 Abs. The expression of IRS-1 and IRS-2 in the cells was detected by WB in 25 μg of whole cell lysates. In (**c**) and (**d**) the control samples were precipitated with carrier beads only (protein A agarose and anti-mouse IgG agarose for IRS molecules and ER-α, respectively), with the omission of the primary Abs, and then processed for WB. (**e**) The expression of ER-α was studied in 50 μg of cytoplasmic lysates before and after IP of IRS-1, IRS-2, or ER-α. The expression of the cytoplasmic protein GAP-DH is shown as a control of loading. The graph represents the expression of each protein relative to the expression of GAP-DH

Re-expression of ER-α coincides with better cell survival in the presence of IGF-I

Previous results suggested that re-expression of ER-α decreases IRS-1 and IRS-2 turnover. Here, we addressed the biologic consequences of this phenomenon. Growth profiles confirmed our previous observations that MDA-MB-231 cells die in SFM in the presence or absence of IGF-I (Bartucci *et al.*, 2001); however, compared with the parental cells, MDA-MB-231/ER cells exhibited significantly better survival in the presence of IGF-I (Figure 5a). This effect coincided with increased IGF-I-dependent tyrosine phosphorylation of IRS-1, increased activity of Akt, and enhanced serine phosphorylation of GSK3 α/β (at 15 min and 1 day of treatment). The upregulation of the Akt/GSK-3 pathway in the presence of ER-α was noted in several experiments and was statistically significant. The increased activation of Akt was paralleled by down-regulation of ERK1/2 at 1 day (Figure 5b), which was in agreement with previously published observations obtained with this and other cellular models (Rommel *et al.*, 1999; Zimmermann and Moelling, 1999; Bartucci *et al.*, 2001). Interestingly, in MDA-MB-231/ER cells, IRS-2 tyrosine phosphorylation was not stimulated upon IGF-I treatment (data not shown), confirming findings obtained in other breast cancer cell models (Jackson *et al.*, 1998).

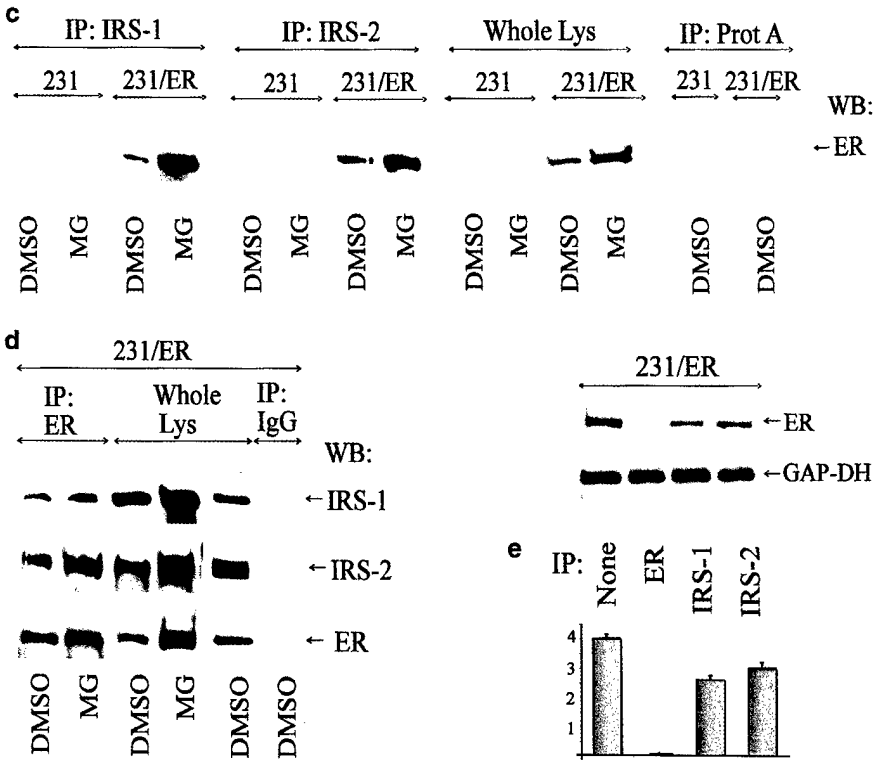

Figure 4 *Continued.*

Discussion

IRS-1 and IRS-2 are expressed in the majority of breast cancer cell lines and have been identified in most breast tumor samples (Jackson *et al.*, 1998; Lee *et al.*, 1999; Molloy *et al.*, 2000; Surmacz, 2000; Jackson *et al.*, 2001; Sachdev and Yee, 2001). In ER-α-positive breast cancer cells, IRS-1, through the activation of downstream kinases PI-3 and ERK1/2, mediates mitogenic signaling of IGF-I (Surmacz, 2000; Sachdev and Yee, 2001), whereas the function of IRS-2 is unclear. Recent data suggested that IRS-2 might be necessary to transmit nonmitogenic signals of IGF-I, such as stimulation of cell migration (Jackson *et al.*, 2001).

The expression and activation of ER-α has been shown to enhance insulin and IGF-I mitogenicity (Lee *et al.*, 1999; Surmacz, 2000; Mauro *et al.*, 2001; Sachdev and Yee, 2001). It is now well established that one important mechanism by which ER-α amplifies the effects of IGF-I and insulin is upregulation of the expression and function of IRS-1 (Lee *et al.*, 1999; Molloy *et al.*, 2000; Surmacz, 2000; Mauro *et al.*, 2001; Sachdev and Yee, 2001). For instance, in MCF-7 breast cancer cells, the activation of ER-α by E2 stimulated the expression of IRS-1 and potentiated IRS-1 signaling through the PI-3K/Akt pathway. These effects were blocked in the presence of antiestrogens (Lee *et al.*, 1999; Salerno *et al.*, 1999; Molloy *et al.*, 2000; Mauro *et al.*, 2001). The upregulation of IRS-1 by E2 has been documented on the transcriptional level (Lee *et al.*, 1999; Molloy *et al.*, 2000; Mauro *et al.*, 2001). E2 did not influence the expression of IRS-3 and IRS-4 mRNAs and proteins, and the stimulation of IRS-2 expression, although noted, has been found statistically insignificant (Molloy *et al.*, 2000).

The regulation of IRS-1 and IRS-2 by ER-α on the post-transcriptional level has never been studied and has been addressed in this work. To distinguish between transcriptional and post-transcriptional effects, we re-expressed ER-α in ER-α-negative MDA-MB-231 cells to develop MDA-MB-231/ER cells. In this cellular model, the presence of ER-α (unliganded) did not affect the expression of IRS-1 and IRS-2 mRNAs.

The experiments with MDA-MB-231/ER cells yielded novel information on the interaction between ER-α and IRS substrates. Especially, we found that (1) ER-α re-expression coincided with higher basal levels of IRS-1 and IRS-2; (2) under SFM conditions, the amounts of IRS-1 and IRS-2 declined only in the absence of ER-α; (3) re-expression of ER-α increased IRS-1 and IRS-2 half-life but did not affect IRS-1 and IRS-2 mRNA levels; (4) in MDA-MB-231/ER cells, degradation of IRS-1, IRS-2, and ER-α proceeded through common proteolytic pathways and a fraction of IRS-1 and IRS-2 was found to associate with ER-α; (5) ER-α re-expression was paralleled by reduced ubiquitination of IRS-1 and IRS-2; (6) higher levels of IRS-1 in MDA-MB-231/ER cells improved long-term activation of the IRS-1/Akt pathway and cell survival in IGF-I.

In MDA-MB-231/ER cells, the addition of E2 significantly reduced ER-α levels indicating that re-expressed ER-α could be liganded and then targeted for degradation, as normally observed in ER-α expressing cells (Nawaz *et al.*, 1999; Lonard *et al.*, 2000). The

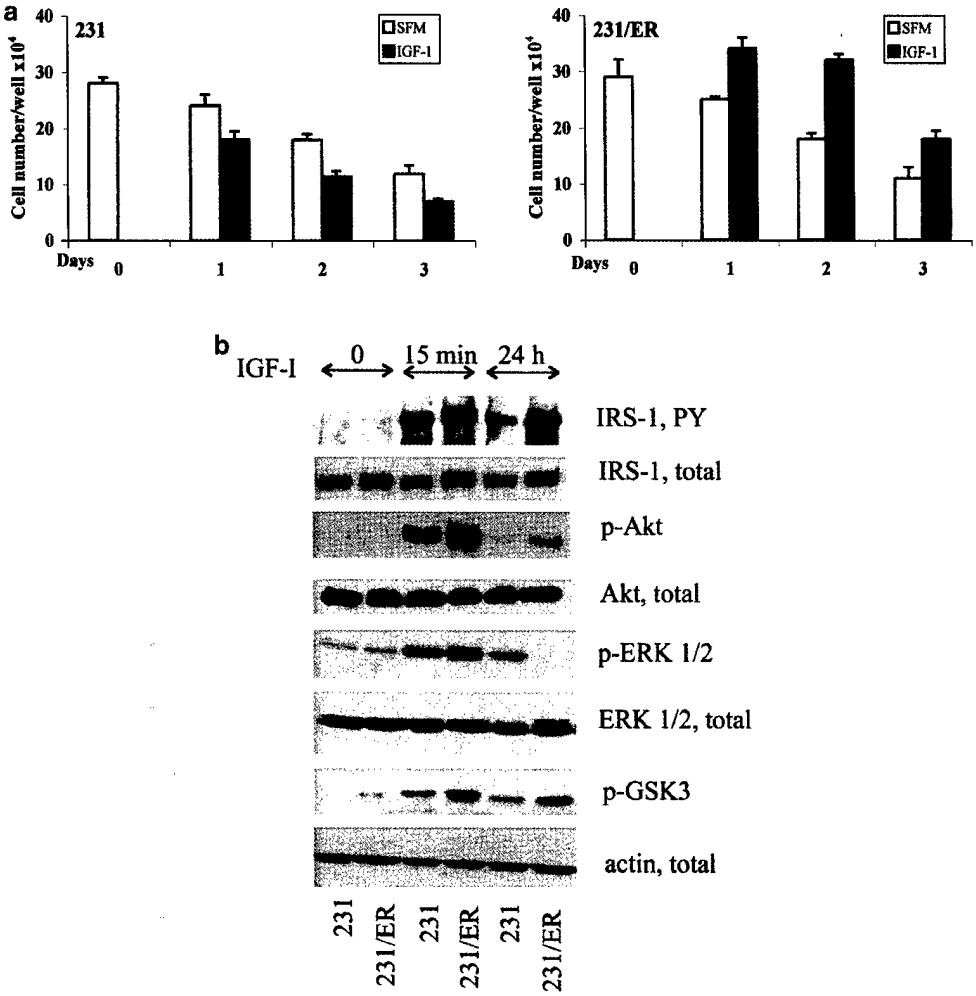

Figure 5 Re-expression of ER-α in MDA-MB-231 cells coincides with better survival in the presence of IGF-I. MDA-MB-231 and MDA-MB-231/ER were synchronized in PRF-SFM and treated with IGF-I as described in Materials and methods. The graphs demonstrate cell numbers at 0, 1, 2, and 3 days of treatment. Growth assays were repeated three times, SE values are shown as error bars (**a**). Tyrosine phosphorylation of IRS-1 was measured by IP and WB at 0, 15 min, and 1 day of IGF-stimulation. The phosphorylation of Akt, GSK3, and ERK1/2 and total levels of Akt, ERK1/2, β-actin were assessed at the same time points by WB in 50 μg of whole-cell lysates using Abs described in Materials and methods (**b**)

degradation of the liganded ER-α has been described to occur through the 26S proteasome, whereas under basal conditions *in vitro*, the degradation of ER-α may involve calpain pathways (Murayama *et al*., 1984; Shiba *et al*., 1996; Nawaz *et al*., 1999; Lonard *et al*., 2000). Our observations that E2 stimulated degradation of ER-α were consistent with other reports. However, we noticed that the degradation of ER-α both ligand-dependent and -independent was reduced in the presence of 26S proteasome and calpain inhibitors, implicating both pathways hi these processes. The differences could reflect the experimental models, as proteasomal degradation of ER-α has been documented in transfected HeLa cells, and calpain proteolysis has been described in the case ER-α purified from porcine uterus (Murayama *et al*., 1984; Nawaz *et al*., 1999; Lonard *et al*., 2000).

Since re-expression of ER-α coincided with greater abundance and stability of IRS-1 and IRS-2 proteins, but not with increased transcription of the corresponding mRNAs, we investigated whether ER-α inhibits IRS-1 and IRS-2 degradation. In our experimental model, IRS-1 and IRS-2 were processed through the 26S proteasome and calpain pathways, which corroborated results obtained with other models (Smith *et al*., 1996; Sun *et al*., 1999; Lee *et al*., 2000; Zhang *et al*., 2000; Pederson *et al*., 2001). Since ER-α is processed through the same mechanisms, we hypothesized that re-expressed ER-α could downregulate the degradation of IRS-1 and IRS-2 by sequestration of enzymes or other proteins involved in common proteolytic processes. Our finding that the presence of ER-α inhibits the ubiquitination of IRS-1 and IRS-2 seems to support this possibility. Interestingly, the effects of ER-α were at least partially specific, as re-expression of ER-α did not affect total protein ubiquitination, or the ubiquitination of several other proteins normally degraded through an MG 132-sensitive pathway, for example, β-catenin (Aberle *et al*., 1997), Hsp 90, and Hsp 70 (Ashok *et al*., 2001).

The stabilization of IRS molecules in the presence of ER-α appears to be biologically significant, especially for the IGF-I phenotype. Our data suggest that MDA-MB-231/ER cells, when compared with MDA-MB-231 cells, are characterized by improved cell survival in IGF-I, enhanced tyrosine phosphorylation of IRS-1, and improved activation of the downstream Akt/GSK-3 pathway. We speculate that the restoration of this pathway was incomplete because some IGF-I effects that are characteristic for normal ER-α-positive cells (e.g., vigorous growth in IGF-I or IGF-IR/PI-3K-dependent degradation of IRS-1) were not observed. The lack of IRS-2 activation by IGF-I confirmed findings in other breast cancer cells (Jackson *et al.*, 1998).

Since the IRS-1, IRS-2, and ER-α are degraded by common mechanisms, we analysed, using several different techniques, whether these proteins can be found in one cellular compartment. By confocal microscopy and subcellular protein fractionation, we determined that in unstimulated MDA-MB-231/ER cells, IRS-1, IRS-2, as well as a fraction of ER-α are found in the cytoplasm. The cytoplasmic presence of these molecules has been additionally confirmed by immunocytochemistry (data not shown). Our confocal microscopy and immunoprecipitation experiments suggested a physical interaction between ER-α and the IRS proteins. The association of ER-α with cytoplasmic signaling molecules is not unusual; for instance, ER-α has been shown to interact with the IGF-IR (Kahlert *et al.*, 2000), PI-3 K (Simoncini *et al.*, 2000; Sun *et al.*, 2001), and SHC (Song *et al.*, 2002), in all cases, enhancing the signaling potential of these molecules in cell stimulated with growth factors (Simoncini *et al.*, 2000; Sun *et al.*, 2001). Our experiments as well as data from other laboratories (Simoncini *et al.*, 2000; Sun *et al.*, 2001) suggest that only a fraction of ER-α interacts with cytoplasmic signaling proteins. The immunoprecipitation and microscopic techniques suggested that approximately 20–30% of ER-α can associate with IRS molecules. Our recent experiments indicate that the ER-α:IRS-1 and ER-α:IRS-2 complexes are not unique to MDA-MB-231/ER cells as they also exist in MCF-7 cells and other ER-α-positive breast cancer cell lines and are regulated by E2 (Morelli *et al.*, unpublished data).

In summary, the expression of ER-α could decrease proteolytic turnover of IRS molecules. We hypothesize that ER-α:IRS-1/2 complex formation represents one of the stages of the common degradation processes. The post-transcriptional interactions between ER-α and IRS-1 exemplify a new aspect of ER/IGF-IR crosstalk and a possible target in breast cancer therapy.

Materials and methods

Plasmids

The pcDNA3-ER expression plasmid (obtained from Dr Diego Sisci, University of Calabria, Italy) encodes the wild-type ER-α under the CMV promoter and contains a neomycin resistance gene.

Cell lines

MDA-MB-231 cells were obtained from ATCC. MDA-MB-231/ER clones were developed by stable transfection of MDA-MB-231 cells with the plasmid pcDNA3-ER using Fugene 6 transfection reagent (Roche) (DNA:Fugene 6 ratio was 1 μg:3 μl). Transfectants resistant to 2 mg/ml G418 (Gibco) were screened for ER-α expression by Western blotting (WB) using an anti-ER-α mouse monoclonal antibody (mAb) F-10 0.2 μg/ml (Santa Cruz). To avoid clonal variation, we used a mixed population of five ER-α-expressing clones (referred to as MDA-MB-231/ER cells).

Cell culture

MDA-MB-231 cells were grown in DMEM:F12 containing 5% calf serum (CS). MDA-MB-231/ER cells were cultured in DMEM:F12 plus 5% CS plus 500 μg/ml G418. In the experiments requiring E2- and serum-free conditions, the cells were cultured in phenol red-free serum-free medium (SFM) (Guvakova and Surmacz, 1997; Salerno *et al.*, 1999).

Growth curves

The cells were plated in six-well plates at a concentration of $1.5–2.0 \times 10^5$ cells/plate in normal growth medium. The following day (day 0), the cells at approximately 50% confluence were shifted to SFM containing 50 ng/ml IGF-I. Cell number was determined at days 0, 1, 2, and 3. A fresh dose of IGF-I was added each day.

RT–PCR

Total cellular RNA was extracted from cells cultured for 0, 1, and 3 days using RNeasy Mini Kit (Qiagen). RNA (1 μg) was reverse transcribed (RT) and then amplified by PCR to obtain products corresponding to cDNA fragments of IRS-1, IRS-2, or β-actin. RT–PCR was performed using the Superscript First Strand synthesis system (Gibco) and PCR Core kit (Roche). The following primers were used: IRS-1 upstream primer 5′-TCCACTGTGACACCAGAATAAT-3′ (nt 4979–5000, human IRS-1 cDNA; Araki *et al.*, 1993), IRS-1 downstream primer 5-CGCCAACATTGTTCATTCCAA-3′ (nt 5721–5741), IRS-2 upstream primer 5′-GCTGCTGCTACAGCTCCT-3′ (nt 2399–2414, human IRS-2 cDNA; Vassen *et al.*, 1999), IRS-2 downstream primer 5′-GGCTCGCCAAAGTCGATGT-3′ (nt 2762–2780), β-actin upstream primer 5′-TGGGAATGGGTCAGAAGGACT-3′ (nt 224–244, human actin cDNA; Powzaniuk *et al.*, 2001), β-actin downstream primer 5′-TTTCACGGTTGGCCTTAGGGTT-3′ (nt 411–433). PCR was performed in a GeneAmp PCR System 9600 thermocycler (Perkin-Elmer) using the following conditions: for IRS-1, 1 min at 94°C, 1 min at 50°C, 2 min at 72°C; for IRS-2, 30 s at 94°C, 30 s at 48°C, 40 s at 72°C; for β-actin, 1 min at 94°C, 1 min at 50°C, 2 min at 72°C. The amplification products obtained in 15, 25, and 35 cycles were analysed in a 1% agarose gel.

Treatment with inhibitors of protein degradation

Confluent cultures (70%) were shifted to SFM and treated for 24 h with MG132 and calpastatin in the presence or absence of E2. MG132 (carbobenzoxy-l-leucyl-L-leucyl-L-leucinal) (Calbiochem) is an inhibitor of the 26S proteasome and was used at the concentration of 10 μM. Calpastatin (a 27 residue peptide encoded by exon 1B of human calpastatin) (Calbiochem) is an inhibitor of calpains I and II, and was used at the concentration 20 nM. E2 (Sigma) was used at the concentration

10 nM. Control cultures were treated with 7 mM DMSO (ICN Biomedicals).

Immunoprecipitation and WB

Cell cultures (70%) were shifted to SFM for 0–72 h and then lysed. Cytoplasmic protein lysates were obtained with a buffer containing 50 mM HEPES, pH 7.5, 150 mM NaCl, 1% Triton X-100, 1.5 mM $MgCl_2$, EGTA 10 mM pH 7.5, glycerol 10%, inhibitors (2 μM Na_3VO_4, 1% PMSF, 20 μg/ml aprotinin). Following the collection of cytoplasmic proteins, the nuclei were lysed with the buffer containing 20 mM KOH–HEPES pH 8, 0.1 mM EDTA, 5 mM $MgCl_2$, 0.5 M NaCl, 20% glycerol, 1% NP-40, and inhibitors (as above). The expression of target proteins was analysed by WB using 25–50 μg of cell lysate and/or by IP in 500 μg–1 mg of lysate. The following antibodies (Abs) were used: anti-IRS-1 polyclonal Ab (pAb) (UBI) for WB and IP; anti-IRS-2 pAb (UBI) for WB and IP; anti-ER-α mAb (Santa Cruz) for WB and IP; anti-β-catenin mAb (Transduction Laboratories) for WB and IP; anti-β-actin mAb (Sigma) for WB, anti-ubiquitin mAb (Santa Cruz) for WB; anti-Hsp 90 mAb (Santa Cruz) for WB and IP; anti-active Akt (Ser473) pAb (Cell Signaling) for WB; anti-total Akt pAb (Cell Signaling) for WB; anti-phospho-p44/42 MAP kinase (T202/Y204) mAb (Cell Signaling) for WB; anti-active GSK3 α/β (Ser21/9) pAb (Cell Signaling) for WB, anti-GAP-DH mAb (Research Diagnostics Inc.) for WB, anti-c-Jun pAb (Santa Cruz) for WB. In all IPs, protein lysates were incubated in HNTG buffer (20 mM HEPES, pH 7.5, 150 mM NaCl, 0.1% Triton X-100, 10% glycerol, and 0.2 mM Na_3VO_4) at 4°C for 4 h with the primary antibodies, and then agarose beads conjugated with Protein A (Calbiochem) (for IP of pAbs) or anti-mouse IgG (Sigma) (for IP of mouse mAbs) were added for another 1 h. In control samples, the primary Abs were omitted. The immunoprecipitated proteins were washed three times with the HNTG buffer, separated by SDS–PAGE (polyacrilamide gel elecrophoresis), and analysed by WB and ECL chemiluminescence (Amersham). The intensity of bands representing relevant proteins was measured by the Scion Image laser densitometry scanning program.

Pulse-chase labeling

To determine the half-life of IRS molecules, we followed the methodology described by Lee *et al.* (2000) and Zhang *et al.* (2000) with some modifications. Briefly, 70% cultures were shifted to methionine- and cysteine-free DMEM (Gibco) for 16 h and then metabolically labeled with ^{35}S (100 μCi/1 ml, Express protein labeling mix, Perkin-Elmer) for 1 h. After that, the labeling medium was replaced with SFM. The cells were lysed at 2, 4, 8, and 12 h to obtain cytoplasmic proteins. The protein lysates were precipitated with anti-IRS-1 and IRS-2 Abs for 4 h and IPs were separated by SDS–PAGE as described above. Labeled IRS molecules were identified by autoradiography.

Confocal microscopy

Confluent cultures (50%) were fixed in 3% paraformaldehyde, permeabilized with 0.2% Triton X-100, washed 3 × with PBS, and incubated for 1 h with primary antibodies (anti-IRS-1 CT (UBI) 2 μg/ml and anti-ER-α F-10 (Santa Cruz) 2 μg/ml), then washed with PBS 3 ×, and incubated with secondary Abs. A fluoresceine-conjugated donkey anti-mouse IgG (Calbiochem) was used as a secondary Ab for ER-α and a rhodamine-conjugated donkey anti-rabbit IgG (Calbiochem) was used for IRS-1. The cellular localization of IRS-1 and ER-α was studied with Bio-Rad MRC 1024 confocal microscope connected to a Zeiss Axiovert 135 M inverted microscope. The optical sections were taken at the central plane. The fluorophores were imaged separately to ensure no excitation/emission wavelength overlap.

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REVIEW

Growth factor receptors as therapeutic targets: strategies to inhibit the insulin-like growth factor I receptor

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Neoplastic transformation is often related to abnormal activation of growth factor receptors and their signaling pathways. The concept of targeting specific tumorigenic receptors and/or signaling molecules has been validated by the development and successful clinical application of drugs acting against the epidermal growth factor receptor 2 (HER2/neu, Erb2), the epidermal growth factor receptor 1 (EGFR, HER1), the Brc-Abl kinase, the platelet-derived growth factor receptor, and c-kit. This review will focus on the next promising therapeutic target, the insulin-like growth factor I receptor (IGF-IR). IGF-IR has been implicated in a number of neoplastic diseases, including several common carcinomas. From a pharmaceutical standpoint, of particular importance is that IGF-IR appears to be required for many transforming agents (genetic, viral, chemical) to act, but is not obligatory for the function of normal adult cells. The tumorigenic potential of IGF-IR is mediated through its antiapoptotic and transforming signaling, and in some cases through induction of prometastatic pathways. Preclinical studies demonstrated that downregulation of IGF-IR reversed the neoplastic phenotype and sensitized cells to antitumor treatments. The strategies to block IGF-IR function employed anti-IGF-IR antibodies, small-molecule inhibitors of the IGF-IR tyrosine kinase, antisense oligodeoxynucleotides and antisense RNA, small inhibitory RNA, triple helix, dominant-negative mutants, and various compounds reducing ligand availability. The experience with these strategies combined with the knowledge gained with current antigrowth factor receptor drugs should streamline the development of anti-IGF-IR therapeutics.
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Antigrowth factor receptor therapeutics

Several growth factor receptors with intrinsic tyrosine kinase activity have been implicated in the development and progression of neoplastic diseases. Growth factor receptor-driven tumorigenicity may be related to increased proliferation, suppression of apoptosis, especially under anchorage-independent conditions, and activation of processes favoring metastatic spread. Consequently, growth factor receptors became a rational target for therapeutic intervention (Shawver *et al.*, 2002). Such target-directed therapy is hoped to become an alternative to conventional nonselective treatments producing undesirable effects in normal cells. Various strategies have been pursued to inhibit tumorigenic effects of growth factors. Two approaches, that is, blocking target receptors by abolishing ligand/receptor binding and inactivating receptor tyrosine kinases with small-molecule inhibitors, provided the basis for the development of current antigrowth factor receptor drugs. The development, mechanisms of action, and clinical characteristics of these new pharmaceuticals have been extensively reviewed in recent publications (Mendelsohn and Baselga, 2000; Druker, 2002; Levitzki, 2002; Shawver *et al.*, 2002; Arteaga, 2003; Fry, 2003). Some of the target therapeutics are listed below as a reference for further discussion on the strategies to inactivate the insulin-like growth factor I receptor (IGF-IR).

The first antireceptor drug has been developed against the epidermal growth factor receptor 2 (HER2/neu, Erb2). HER2 is a type I transmembrane tyrosine kinase amplified in many cancers, including breast cancer, ovarian cancer, and stomach cancer (Mendelsohn and Baselga, 2000; Holbro *et al.*, 2003). HER2 overexpression typically is associated with more aggressive behavior and poor clinical outcome. In invasive breast cancer, high amplification of HER2 is seen in 25–30% of cases. More than a decade of research and clinical trials led to the launch of the anti-HER2 drug, Herceptin (Trastuzumab; Genentech), in 1998. Herceptin is a humanized monoclonal antibody (MAb) against HER2 and is indicated for the treatment of patients with metastatic breast cancer, whose tumors overexpress HER2. Herceptin is used both as first-line treatment in combination with paclitaxel, and as a second- and third-line therapy (Harries and Smith, 2002; Shawver *et al.*, 2002). The extension of these applications to early breast cancer and other neoplastic diseases is being evaluated (www.clinicaltrials.gov).

The EGFR (HER1) and its ligands are often overexpressed in many types of human tumors (Mendelsohn and Baselga, 2000; Arteaga, 2003). The therapeutic inhibition of EGFR has been achieved with two classes

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of compounds: humanized Abs binding and down-regulating the receptor and small inhibitors inactivating the EGFR tyrosine kinase by blocking ATP binding site (Fry, 2003). Presently, three humanized EGFR Abs are evaluated in clinical trials (Shawver *et al.*, 2002; Arteaga, 2003). Of these, the best developed is Cetuximab (erbitux, C225; Imclone), which is now under clinical assessment for the therapy of advanced colorectal cancer, squamous cell carcinoma, and pancreatic cancer (www.clinicaltrials.gov). Also, several small-molecule inhibitors of EGFR have entered the late stages of clinical development (Shawver *et al.*, 2002; Fry, 2003). One of them, Iressa (gefitinib, ZD1839; AstraZeneca), is a quinazoline competing with ATP for the binding site in the EGFR tyrosine kinase domain. Iressa has been already approved in Japan for the treatment of non-small-cell lung cancer (NSCLC) and its efficacy as a single agent or in combination with other therapies to treat NSCLC and other cancers is under evaluation in the US clinical trials (www.clinicaltrials.gov).

Another successful antityrosine kinase drug is Gleevec (imatinib mesylate, STI571; Novartis). Gleevec is a small-molecule inhibitor blocking the Bcr-Abl cytoplasmic tyrosine kinase that is constitutively active in patients with chronic myelogenous leukemia (CML), and in 15–30% adults with acute lymphoblastic leukemia. Gleevec is also active against the tyrosine kinases of the platelet-derived growth receptor and c-kit, which are often hyperactivated in brain tumors and gastrointestinal stromal tumors (GIST), respectively (Shawver *et al.*, 2002). Currently, Gleevec is indicated for the treatment of CML and advanced GIST carrying an activating c-kit mutation, and its possible use against other malignancies is under investigation (Hernandez-Boluda and Cervantes, 2002; Druker, 2002; www.clinicaltrials.gov). Other recently developed antigrowth factor receptor compounds include inhibitors of the tyrosine kinase of the vascular endothelial growth factor receptor 2 (VEGFR2). These compounds appear to reduce tumor neovascularization, halting its enlargement and expansion (Shawver *et al.*, 2002).

The clinical success with Herceptin, Iressa, and Gleevec validates the concept of antigrowth factor therapy and justifies the pursuit of strategies against similar targets. This review will focus on the next promising pharmaceutical target, IGF-IR.

IGF-IR and its signaling

IGF-IR is an evolutionary conserved, ubiquitous transmembrane tyrosine kinase structurally similar to the insulin receptor (IR) (Ullrich *et al.*, 1986). IGF-IR is composed of two extracellular alpha subunits and two intracellular beta subunits (Figure 1). The alpha subunits bind ligands (IGF-I, IGF-II, and insulin at supraphysiological doses), while beta subunits transmit ligand-induced signal. The beta subunits contain three major domains: the juxtamembrane domain, tyrosine kinase domain, and the C-terminus (Figure 1). The tyrosine kinase domain shares high (~85%) homology with its counterpart in IR, while the C-terminus is only ~40% homologous with the C-terminus of IR (Ullrich *et al.*, 1986).

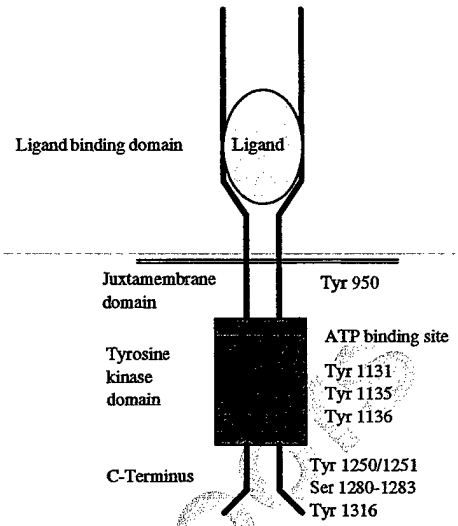

Figure 1 Structure of IGF-IR. Major domains and critical residues are indicated (details in the text)

Binding of ligands to IGF-IR induces its autophosphorylation and tyrosine phosphorylation of IGF-IR substrates, especially the IR substrate 1 (IRS-1) and src- and collagen-homology (SHC) protein. Tyrosine-phosphorylated IRS-1 and SHC bind different effector proteins (enzymes and/or adapters) inducing multiple signaling cascades, among them several interconnecting pathways controlling cell survival and proliferation (Shepherd *et al.*, 1998; White, 1998, 2002; Adams *et al.*, 2000; O'Connor *et al.*, 2000; Surmacz, 2000). The critical survival pathway activated by IGF-I stems from IRS-1. IRS-1 recruits and stimulates the PI-3 kinase (PI-3K), which then transmits signal to the serine/threonine kinase Akt (Akt). Activated Akt phosphorylates and blocks a variety of proapoptotic proteins, including BAD, caspase-9, forkhead transcription factors, and the GSK-3 beta kinase. Furthermore, Akt induces the expression of antiapoptotic proteins, for example, Bcl-2 (Brazil *et al.*, 2002; Hill and Hemmings, 2002; Nicholson and Anderson, 2002). Other mitogenic/survival IGF-IR pathways involve signal transducers and activators of transcription (STATs) that are phosphorylated and activated by IGF-I through JAK1/2 and PI-3K/Akt pathways (Zong *et al.*, 1998, 2000). In addition, IGF-IR can prevent cell death or induce proliferation via the SHC/Ras/ERK1/2 pathway (Peruzzi *et al.*, 1999).

While antiapoptotic and growth pathways of IGF-IR have been extensively studied, the signals controlling nonmitogenic functions of IGF-IR, such as cell–substrate adhesion, migration, invasion, or intracellular

interactions are less well understood. There is increasing evidence that IGF-IR pathways interconnect with integrin and cadherin signaling systems (Vuori and Ruoslahti, 1994; Guvakova and Surmacz, 1999; Mauro *et al.*, 1999, 2003; Reiss *et al.*, 2001a; Shaw, 2001). In some experimental models, IGF-IR has been shown to mediate metastasis, possibly through enhanced migration (Doerr and Jones, 1996; Bartucci *et al.*, 2001), reduced cell–cell adhesion (Mauro *et al.*, 2003), and upregulation of plasminogen activator uPA and matrix metalloproteinases (Long *et al.*, 1998; Dunn *et al.*, 2001; Zhang and Brodt, 2003).

IGF-IR structure–function studies

The extensive mutational analysis of IGF-IR identified receptor domains required for the initiation of specific pathways and linked these pathways with specific functions, that is, proliferation (measured as cell growth in monolayer), survival (usually measured as the ability of cells to survive under anchorage-independent conditions), and transformation (assessed as the ability to grow in soft agar or to form foci). The experiments using different cell models unequivocally demonstrated that the mutation in the ATP binding site of the IGF-IR tyrosine kinase domain produced 'dead' receptors incapable of signal transmission (Figure 1). Mutations at other residues of the tyrosine kinase impaired IGF-IR only partially. For instance, the substitutions of tyrosines (Tyr) 1131, 1135, and 1136 into phenylalanines abrogated transforming signaling and mitogenesis, but not survival signaling. Mutations in either Tyr 1131 or Tyr 1135 downregulated transformation without reducing cell growth. Tyr 950 in the IGF-IR juxtamembrane domain was found necessary for IRS and SHC binding, and for induction of mitogenic and transforming activity, but the IGF-IR/Tyr 950 mutant still transmitted antiapoptotic signaling, confirming that in addition to the classic IRS-1-dependent PI-3K/Akt pathway, other survival pathway(s) emanate from IGF-IR (Hongo *et al.*, 1996; O'Connor *et al.*, 1997; O'Connor, 1998; Romano *et al.*, 1999).

Deletion of the entire C-terminus at aa 1229 totally abrogated transforming function, without inhibiting mitogenic and antiapoptotic ability (Surmacz *et al.*, 1995). The 'transforming domain' was mapped between residues 1245 and 1310, with Tyr 1251, Ser 1280–1283, His 1293, and Lys 1294 required for transformation (Hongo *et al.*, 1996) (Figure 1). It is worth nothing that C-terminal deletions (at residue 1229 or 1245) appeared to amplify antiapoptotic effects, suggesting that the C-terminus acts as an intrinsic inhibitor of IGF-IR survival signaling. The mutations in the C-terminus at Tyr 1250/1251, His 1293, and Lys 1294 reduced survival, implying that these residues act as neutralizers of the C-terminus proapoptotic function (Hongo *et al.*, 1996; O'Connor, 1998).

The important practical implication of the above studies is that transformation by IGF-IR does not occur without activated IGF-I survival pathways. Thus, targeting the survival function of IGF-IR should be the optimal approach to inhibit tumorigenicity. As evidenced by the mutational analysis, the best way to achieve this affect is to inactivate totally the IGF-IR tyrosine kinase.

IGF-IR and tumorigenesis

Initially, the importance of IGF-IR in tumorigenesis was suggested by the observation that constitutive overexpression of IGF-IR and/or hyperactivation of its signaling induced the transformed phenotype in cultured cells (Kaleko *et al.*, 1990; Pietrzkowski *et al.*, 1992a). Later experiments with cells derived from IGF-IR knockout mice provided evidence that different tumorigenic agents (viral, chemical, genetic) were not able to induce transformation in the absence of IGF-IR, but were transforming when IGF-IR (but not IR) was re-expressed (Baserga, 1994, 1995, 1998). This controlling function of IGF-IR most likely depends on its ability to counteract strong susceptibility to apoptosis observed in transformed cells (Baserga, 1996, 1997, 1998). Additional important observation stemming from the studies on IGF-IR knockout cells and normal human fibroblasts was that normal, untransformed cells are more resistant to apoptosis and can survive, or even proliferate (albeit at slower rate), in the absence of functional IGF-IR (Baserga, 1998). Thus, transformed but not normal cells should be especially sensitive to anti-IGFIR drugs.

In vitro experiments with tumor cell lines and epidemiological studies provided evidence that activation of IGF-IR is implicated in the development of many common neoplastic diseases, including carcinomas of lung, breast, prostate, pancreas, liver, and colon (Korc, 1998; Pollak, 2000; Surmacz, 2000; Djavan *et al.*, 2001; Giovannucci, 2001; Sachdev and Yee, 2001; Scharf *et al.*, 2001; Druckmann and Rohr, 2002). In some diseases, for instance in primary breast cancer, IGF-IR is overexpressed and hyperphosphorylated compared with its status in normal mammary epithelial cells (Surmacz, 2000). Hyperactivation of IGF-IR may result from autocrine or paracrine stimulation, as many tumors produce IGF-IR ligands (Sachdev and Yee, 2001). In addition, high abundance of endocrine IGFs may enhance IGF-IR signal. In this context, it is worth noting that elevated plasma concentrations of IGF-I have been linked with increased risk of several types of cancer (Pollak, 2000). Similarly, data from experimental systems demonstrated that elevated IGF-I levels correlate with increased tumorigenicity in experimental models (Dunn *et al.*, 1997; Butler *et al.*, 1998).

Targeting IGF-IR and its signaling

IGF-IR fulfills several criteria of an attractive pharmaceutical target: (1) IGF-IR is implicated in neoplastic transformation; (2) abundance of IGF-IR and its

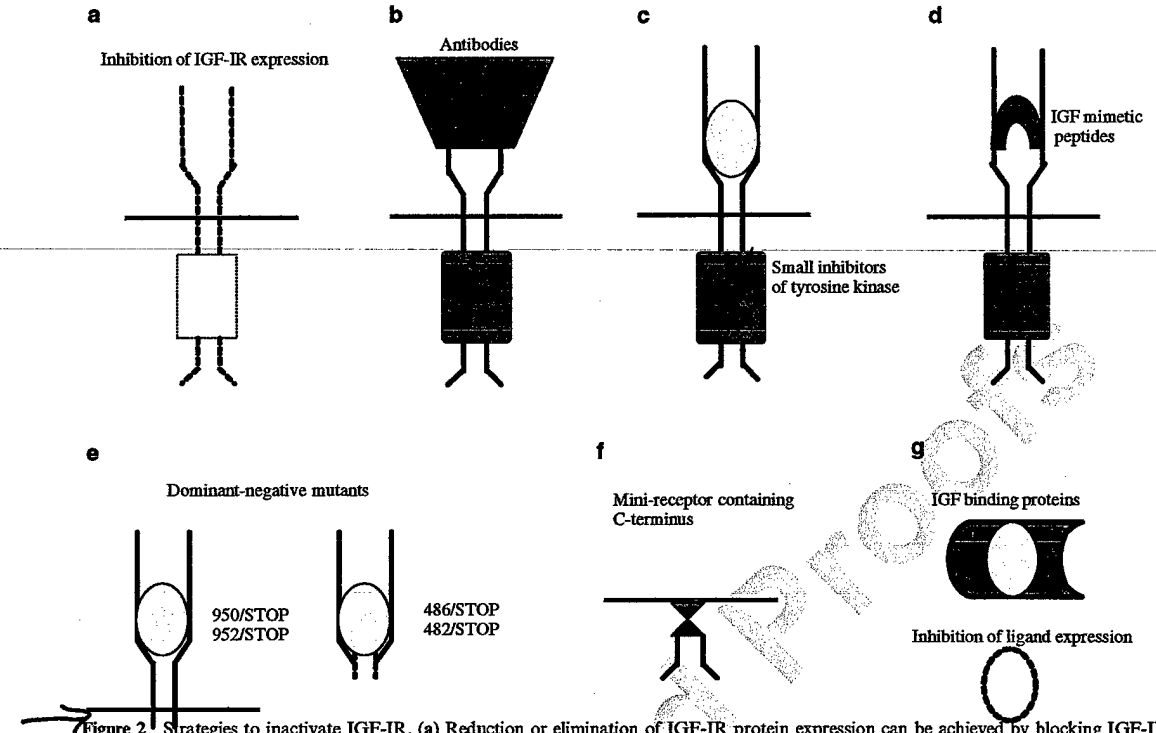

Figure 2 Strategies to inactivate IGF-IR. (a) Reduction or elimination of IGF-IR protein expression can be achieved by blocking IGF-IR transcription (with triple helix) or translation (with antisense oligodeoxynucleotides, antisense RNA constructs, and siRNA). (b) IGF-IR function can be blocked with inactivating Abs. Binding of Abs to IGF-IR prevents ligand binding and induces receptor degradation. (c) IGF-IR tyrosine kinase activity can be abolished with small-molecule inhibitors. (d) IGF-I mimetic peptides can compete with natural ligands. Their binding to IGF-IR inactivates the receptor. (e) IGF-IR mutants lacking beta-subunits can act as dominant-negative receptors. (f) Expression of myristylated IGF-IR C-terminus, a domain with intrinsic proapoptotic activity, can downregulate survival signals. (g) Ligand availability can be reduced with excess binding proteins, or by inhibition of ligand expression on the transcriptional or post-transcriptional level

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ligands can be easily measured in clinical samples, and (3) *in vitro* studies identified critical parameters for IGF-IR induction. The greatest challenge in targeting IGF-IR is designing strategies that would specifically inhibit IGF-IR without blocking IR and producing diabetogenic effects. The stage for anti-IGF-IR drugs has been set by a variety of laboratory studies, demonstrating that inhibition of either IGF-IR/ligand binding, IGF-IR expression, or IGF-I signaling can exert antitumor effects. Several of these approaches are discussed below.

Antibodies

The initial approach to inhibit IGF-IR signaling was based on the use of IGF-IR blocking antibodies (Figure 2b). The mouse MAb alpha-IR-3 raised against the alpha domain of IGF-IR (Jacobs *et al.*, 1986) inhibited IGF-IR activation and IGF-IR-dependent mitogenicity in several cell types *in vitro*, including breast carcinoma (Arteaga *et al.*, 1989; Arteaga, 1992), rhabdomyosarcoma (Kalebic *et al.*, 1994), NSCLC (Zia *et al.*, 1996), and Ewing's sarcoma (Scotlandi *et al.*, 1998). However, in some cases alpha-IR-3 was ineffective in blocking IGF-I-sensitive tumors in animal models (Arteaga, 1992). Furthermore, it has been reported that alpha-IR-3 may exhibit agonistic abilities towards IGF-IR (De Leon *et al.*, 1992; Kato *et al.*, 1993).

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The mouse anti-IGF-IR MAb 391 inhibited IGF-IR autophosphorylation and signaling to Akt in several human cancer cell lines. Chronic treatment with MAb 391 resulted in downregulation of receptors through lysosome-dependent pathways (Hailey *et al.*, 2002). Several other mouse anti-IGF-IR MAbs were described (Li *et al.*, 1993, 2000). One of them, MAb 1H7, which blocks IGF-IR/IGF-I binding and IGF-IR-dependent DNA synthesis was used to engineer a single-chain humanized anti-IGF-IR scFv-Fc Ab and contains the Fc domain of human IgG1 fused to the Fv region of 1H7 (Li *et al.*, 2000). Treatment of MCF-7 breast cancer cells with scFv-Fc for 2–24 h downregulated the levels of IGF-IR through the lysosomal/endocytic pathway, rendering the cells refractory to IGF-I stimulation (Sachdev *et al.*, 2003). Importantly, downregulation of IGF-IR by scFv-Fc occurred also in MCF-7 xenografts and was paralleled by reduced tumor growth (Sachdev *et al.*, 2003). These or similar humanized MAbs will

likely become a model for future drug development once their specificity towards IGF-IR and lack of IR cross-reactivity is demonstrated *in vivo*.

Low molecular weight molecules targeting IGF-IR tyrosine kinase

High-throughput technology combined with computer modeling is currently used to identify low molecular weight compounds blocking the IGF-IR tyrosine kinase (Figure 2c). The first described IGF-IR inhibitors, tyrphostins AG 538 and I-OMeAG, were modeled on the IR tyrosine kinase. The compounds inactivated the IGF-IR tyrosine kinase by blocking the substrate binding site; however, crossreactivity with the IR tyrosine kinase was reported (Blum *et al.*, 2000).

Recent advances in the characterization of the three-dimensional structures of IGF-IR and IR greatly facilitated the design of specific IGF-IR inhibitors (De Meyts and Whittaker, 2002). Most importantly, crystallographic studies revealed conformational differences in the phosphorylated forms of IGF-IR and IR kinases, the feature allowing the development of selective therapeutics (Favelyukis *et al.*, 2001; Pautsch *et al.*, 2001). Several new compounds with enhanced specificity towards IGF-IR and low crossreactivity with IR entered into preclinical studies. The examples include derivatives of pyrimidine and podophyllotoxin, disclosed in patent applications WO 02/092599 and WO 02/102804, respectively. Specific small inhibitors of IGF-IR are likely candidates to become anti-IGF-IR drugs. The positive experience with similar therapeutics (Iressa, Gleevec), especially the possibility of oral delivery and low toxicity, makes this approach especially attractive.

IGF-I mimetic peptides

A series of small IGF-I peptide analogues was designed by molecular modeling of the IGF-I protein (Pietrzkowski *et al.*, 1992b, 1993) to compete with IGF-IR ligands (Figure 2d). The synthetic peptides were modeled on C and D domains of IGF-I, as these domains contain the least similarity between IGF-I and insulin. One of the peptides, JB1 (modeled on the D domain) effectively inhibited IGF-I-dependent IGF-IR autophosphorylation and proliferation in several tumor cell lines. The analogues used at nano- or micromolar concentrations exhibited good specificity for IGF-IR, and low toxicity for cells in cell culture (Pietrzkowski *et al.*, 1992b, 1993). However, the efficacy of these compounds against experimental tumors *in vivo* has never been assessed.

Modulators of IGF-IR internalization and recycling

Following ligand binding, the IGF-IR/ligand complex is internalized, the ligand is degraded by endosomal proteinases, and the receptor is returned to the membrane. One way to reduce IGF-I effects is to block IGF-IR re-expression on the cell surface. Recent studies suggested that IGF-IR trafficking could be substantially blocked by the inhibition of IGF-I-degrading enzymes, for example, cathepsin. The cathepsin inhibitors, E-64- and CA074-methyl ester, reduced IGF-IR expression on the cell surface and impaired several IGF-I-dependent effects, including DNA synthesis, cell survival, anchorage-independent growth, and synthesis of matrix metalloproteinases in human breast cancer and murine lung carcinoma cells (Brodt *et al.*, 2000; Navab *et al.*, 2001).

Antisense nucleotides, antisense RNA, siRNA, triple helix

A variety of experiments employing antisense oligodeoxynucleotides (ODNs), antisense RNA, and small interfering RNA (siRNA) demonstrated that IGF-IR-dependent tumorigenicity can be decreased or eliminated by blocking IGF-IR mRNA, thus inhibiting IGF-IR protein synthesis (Figure 2a). Most of the reported anti-IGF-IR ODNs contained sequences complementary to the IGF-IR translation initiation site. The association of these reagents with IGF-IR mRNA produced heteroduplex that was cleaved by RNase H. Multiple studies documented that anti-IGF-IR ODNs (regular or phosphorotioate chemistry) at nanomolar concentrations decreased IGF-IR expression, reduced cell proliferation, and induced apoptosis in various human and rodent cancer cell types in cells grown in culture (Pietrzkowski *et al.*, 1993; Resnicoff *et al.*, 1994, 1995a, b; Muller *et al.*, 1998; Coppola *et al.*, 1999; Macaulay *et al.*, 2001; Pavelic *et al.*, 2002; Bohula *et al.*, 2003). Furthermore, in some instances, treatment with ODNs induced massive apoptosis and tumor regression in animal models (Resnicoff *et al.*, 1995a, b). However, the reduction of IGF-IR expression was often incomplete even with high concentrations (100–500 nM) of ODNs, possibly due to their poor association with target sequences (Macaulay *et al.*, 2001). Moreover, interactions of anti-IGF-IR ODNs with IR synthesis were reported (Bohula *et al.*, 2003).

To address these problems, Bohula *et al.* (2003) used scanning oligonucleotide arrays to probe the secondary structure of IGF-IR mRNA in order to identify target sequences that are accessible for ODNs, and do not appear in IR mRNA. This strategy enabled selection of specific ODNs that effectively and selectively downregulated IGF-IR in human cancer cell lines (Bohula *et al.*, 2003). Furthermore, the accessible sequences were suitable targets for anti-IGF-IR siRNAs. Indeed, some of the designed siRNAs were able to silence target IGF-IR mRNA sequences. The effect was paralleled by repression of IGF-IR synthesis and downregulation of IGF-IR signaling (Bohula *et al.*, 2003).

In addition to ODNs and siRNA, different antisense-IGF-IR RNA vectors containing fragments of IGF-IR cDNA cloned in 3′–5′ orientation were generated to

inhibit IGF-IR expression. The vectors (plasmids or viruses) produced antisense RNA that hybridized with complementary sequences in IGF-IR mRNA, blocking IGF-IR synthesis. For instance, an antisense IGF-IR RNA against the first 309 bases of IGF-IR mRNA, delivered to cells by transfection or adenoviral infection, reduced IGF-IR expression, IGF-I-dependent proliferation and survival in a number of human and rodent cell models, including endometrial cancer (Nakamura *et al.*, 2000), Ewing's sarcoma (Scotlandi *et al.*, 2002), and rat glioblastoma (Resnicoff *et al.*, 1994). The expression of this IGF-IR antisense efficiently inhibited tumorigenicity of cells grown as explants in experimental animals, most probably by induction of massive apoptosis (Resnicoff *et al.*, 1994, 1995b; Scotlandi *et al.*, 2002). The same antisense construct inhibited metastasis of murine lung carcinoma cells (Brodt *et al.*, 2000). Another antisense IGF-IR plasmid containing ~300 bp DNA complementary to the region surrounding the IGF-IR translation initiation site was used to inhibit IGF-IR expression and function in breast cancer cells (Neuenschwander *et al.*, 1995).

In human rhabdomyosarcoma cells, transfection of an antisense IGF-IR plasmid containing sequences complementary to the first 700 bp of IGF-IR coding sequence markedly reduced growth rates in monolayer and soft agar and impaired tumor formation in immunodeficient mice (Shapiro *et al.*, 1994). This antisense delivered to human lung cancer cells decreased the expression of IGF-IR, inhibited growth in soft agar, and prolonged the survival of animals bearing established xenografts (Lee *et al.*, 1996). A similar plasmid containing a 697 bp fragment exon 1–3 was effective in decreasing the growth and metastasis of breast cancer cells (Chernicky *et al.*, 2000) and rat prostate cancer cells (Burfeind *et al.*, 1996).

In many cases, the induction of cell death with antisense IGF-IR strategies was much more pronounced *in vivo* (animal models) than *in vitro* (monolayer tissue culture or soft agar), suggesting that *in vivo* tests may be superior in screening for anti-IGF-IR compounds (Resnicoff *et al.*, 1995a, b; Nakamura *et al.*, 2000).

Oligonucleotide-directed triple helix formation is an approach to block transcription of specific genes by inhibiting the passage of RNA polymerase along target DNA. The third effector strand (oligoribonucleotide) contains oligopurine sequences potentially capable of forming a triple helix with oligopurine and/or oligopyrimidine sequenced in target DNA. The triple helix strategy has been reported to be effective in downregulation of IGF-IR. Specifically, a plasmid encoding the homopurine RNA sequences designed to form a triplex with a homopurine·homopyrimidine sequence present 3′ to the termination codon of the IGF-IR gene suppressed IGF-IR transcription in rat C6 glioblastoma cells. The triple helix reagent induced dramatic reduction of IGF-IR transcripts and IGF-IR expression and inhibited tumor formation in nude mice (Rininsland *et al.*, 1997).

Interestingly, in the case of rat C6 glioblastoma and some other cellular models, downregulation of IGF-IR by antisense approaches was associated with the induction of an immune host response leading to elimination of untreated established tumors (Resnicoff *et al.*, 1994; Baserga, 1998;). This peculiar effect, perhaps related to the induction of immune response by the presence of apoptotic cells (Trojan *et al.*, 2002), was further explored in pilot studies involving patients with astrocytomas treated with autologous glioma cells exposed to anti-IGF-IR ODNs (Andrews *et al.*, 2001).

Dominant-negative mutants and mini-receptors

A variety of mutant IGF-IRs with deletions of different sizes and substitutions of critical residues were delivered (by transfection or infection) to IGF-I-sensitive cells to test their dominant-negative potential against wild-type receptors (Figure 2e). Different mutations in the tyrosine kinase domain (ATP binding site, Tyr 1131, 1135, 1136) were reported to reduce IGF-IR-dependent proliferation in monolayer. In addition, mutations in the C-terminus, especially Tyr 1250/1251 inhibited anchorage-independent growth or foci formation, but had only limited effects on cell proliferation (Burgaud *et al.*, 1995; Blakesley *et al.*, 1996; Kalebic *et al.*, 1998; Brodt *et al.*, 2001; Seely *et al.*, 2002). In some experimental models, the mutations in the tyrosine kinase domain and in Tyr 1250/1251 reduced tumor development in animals (Blakesley *et al.*, 1996; Seely *et al.*, 2002), while in other studies these mutations did not exhibit a dominant-negative effect *in vivo* (Burgaud *et al.*, 1995).

More consistent antitumorigenic effects *in vivo* were obtained with IGF-IR mutants containing large truncations. Two mutants encoding only alpha subunits (468/STOP and 482/STOP) reduced tumor growth in animals and produced extensive apoptosis *in vivo*. In addition, the mutants exhibited a bystander effect against the neighboring wild-type cells (D'Ambrosio *et al.*, 1996; Reiss *et al.*, 1998; Adachi *et al.*, 2002; Lee *et al.*, 2003). Furthermore, 468/STOP inhibited experimental metastasis (Dunn *et al.*, 1998). The mechanism of action of these STOP mutants is not fully understood. On the one hand, they are secreted proteins that are able to bind ligands reducing their bioavailability (D'Ambrosio *et al.*, 1996; Lee *et al.*, 2003). On the other hand, 468/STOP has been found in the cytoplasm of the producing cells and reported to inhibit endogenous IGF-IR synthesis (Reiss *et al.*, 2001b). Other two truncated receptors 950/STOP and 952/STOP that lack most of the beta subunit (including the IGF-IR tyrosine kinase) inhibited tumor growth in animals and IGF-IR signaling *in vitro* (Prager *et al.*, 1994; Lee *et al.*, 2003). These receptors most likely block IGF-IR activity by dimerization with endogenous wild-type receptors.

An innovative approach to reduce IGF-IR-dependent survival involved mini-receptors expressing C-terminal domains (Figure 2f). Since deletion of the entire C-terminus enhanced survival, overexpression of this domain was expected to increase apoptosis. Indeed, mini-receptors containing the last 108 aa of IGF-IR

(CF) spliced to the myristylation signal (MyCF) to ensure cell membrane association induced apoptosis in many cell types, sensitized cells to UV irradiation, and abrogated tumorigenesis in nude mice. Furthermore, mutations of MyCF at residues that were previously found to silence proapoptotic activity of the C-terminus reduced its antitumor potential (Hongo *et al.*, 1998; Liu *et al.*, 1998).

Downregulation of IGF-IR ligands

Since high levels of IGF-IR ligands have been implicated in the etiology of many neoplastic diseases, strategies to reduce ligand availability have been developed as possible therapeutics (Figure 2g). The strategies to downregulate ligand expression or availability included the use of ribosymes, RNA enzymes that specifically cleave target RNAs (Guo *et al.*, 2003), triple helix, and antisense RNA (Trojan *et al.*, 2002), and overexpression of IGF-I binding proteins (Yee, 2002).

Combined strategies

The downregulation of IGF-IR has been shown to increase sensitivity to different conventional antitumor treatments (Baserga, 1998). Thus, the use of anti-IGF-IR compounds in combination with other therapies can be envisioned. In addition, cells refractory to other treatments (e.g. anti-HER2 drugs) may present IGF-I sensitivity, thus addition of anti-IGF-IR therapy may improve current antigrowth factor receptor approaches (Lu *et al.*, 2001).

Perspectives for anti-IGF-IR pharmaceuticals

IGF-IR is a promising target in cancer therapy because: (1) IGF-IR expression is easily measurable by conventional techniques; (2) tumor cells may be more sensitive to targeting IGF-IR than normal cells; and (3) IGF-IR is often required for the tumorigenic effects of other oncogenic agents. Thus targeting IGF-IR can be combined with other therapies. Unlike with HER2 and EGFR, the development of anti-IGF-IR pharmaceuticals is still in early discovery phases. Similar to HER2 and EGFR, however, the most advanced strategies are those involving small inhibitors of the IGF-IR tyrosine kinase and anti-IGF-IR antibodies. Other approaches, such as siRNA, antisense, and triple helix strategies are also promising, but they will require optimization of specificity *in vivo* and efficient and safe delivery systems.

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